Medical Library FEB 12 1943

Vol. 36, Nos. 10, 11 & 12 December, 1942

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

C. R. HARINGTON

ASSOCIATE EDITORS

S. J. COWELL

and

F. DICKENS

LONDON
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, N.W. 1

CHICAGO: The University of Chicago Press
(Agents for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan

All rights reserved

1942

Price Fifteen Shillings net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted solely for the advancement of the Science of Biochemistry. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

Members are entitled to receive a copy of the Journal. Further information as to the conditions of membership may be obtained on application to the Hon. Secs., Dr W. T. J. Morgan, Lister Institute, Chelsea Bridge Road, London, S.W.1, or Dr W. Robson, Dept. of Physiology, King's College, Strand, London, W.C. 2., or to the Hon. Treas., Mr J. Addyman Gardner, 35 Bullingham Mansions, Church Street, London, W.8, to whom subscriptions should be sent.

Papers for publication should be sent to Professor F. G. Young, St Thomas's Hospital Medical School, The Manor House, Godalming, Surrey. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued in monthly numbers and the date at which each paper is received is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3. 10*s.* 0*d.* net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1, or to the Hon. Treas., Mr J. Addyman Gardner, 35 Bullingham Mansions, Church Street, London, W. 8. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America.

Notice to Contributors

1. Contributors who are members of the Biochemical Society receive 25 copies (without covers) of their papers free and can purchase additional copies if notice be given when their proofs are returned. Other contributors may purchase reprints but receive no free copies.

2. Papers submitted for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. Contributors residing outside Europe are requested to nominate someone resident in Great Britain to be responsible for the correction of the proofs of their papers. Papers from such contributors should be accompanied by a statement of the number of reprints required.

5. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann, D. (1912). *Z. Biol.* **59**, 17. Titles of Journals should be abbreviated in accordance with the "World List of Scientific Periodicals". In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

6. Chemical formulae should be written, as far as possible, in a single horizontal line.

7. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards or ordinary curve paper in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Legends should be typed on slips of paper attached to the drawings.

81. Note on a Colour Reaction between Ergosterol and Methyldichlor-Arsine

By **P. M. Baranger** and **J. M. Mercier**, *École Polytechnique, Paris* and *Centre de Technologie Scientifique et Coloniale de la France Libre*

(*Received 3 July 1942*)

Several authors have already studied the colour reactions obtained when arsenic trichloride or antimony trichloride react with different ethylenic substances. We have observed that organic derivatives of arsenic, such as $R\mathrm{AsCl_2}$ and $R_1R_2\mathrm{AsCl}$, are equally capable of giving colour reactions with the same substances. We propose to give here the results obtained in the spectrophotometric study of the colour reactions which take place between ergosterol and methyldichlor-arsine.

Variations in the reaction occur according to whether the ergosterol is employed in a concentrated solution and the methyldichlor-arsine in a dilute solution or vice versa. Each of these conditions requires a complete spectrophotometric study if it is desired to use the reaction for the estimation of arsine. We have paid special attention to the case in which a concentrated solution of ergosterol, in chloroform, reacts on a dilute solution of methyldichlor-arsine.

A qualitative study by means of the spectrophotometer shows that this reaction is a function of the concentrations, the temperature and of the time. We utilized a Pulfrich spectrophotometer. If I_0 represents the intensity of a monochromatic beam of light striking a layer of liquid with parallel sides and I the intensity of the same beam after having passed through the liquid, the instrument is calibrated to read directly the extinction coefficient, $E = -\log (I/I_0)$.

If the colours which are produced when chloroform solutions of ergosterol and methyldichlor-arsine react together, are studied, it is noticed that E is a function of the following values: c = concentration of the methyldichlor-arsine (mg./ml.), T = temperature at the time of the experiment (° C), t = time (min.), λ = wavelength of the incident light.

We shall describe the methods employed and give the results of the values obtained when one or more of the factors are allowed to vary, the others being kept constant. The concentration of ergosterol is kept constant throughout all these experiments.

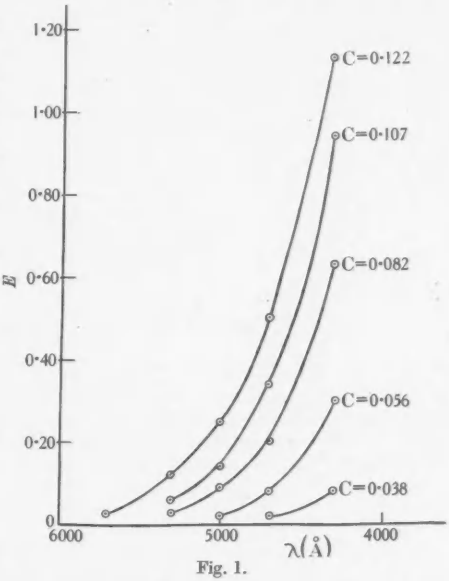


Fig. 1.

Qualitatively, under these experimental conditions, it is seen that a golden yellow tint is obtained when the concentration of the methyldichlor-arsine is in the neighbourhood of 0·040 mg./ml., and that this tint presents in the visible spectrum a maximum of absorption in the violet $\lambda = 4300$ Å., and a maximum transmission from $\lambda = 5700$ Å (see Fig. 1).*

* For stronger concentrations of methyldichlor-arsine a green tint is obtained, corresponding to a new absorption band in the neighbourhood of $\lambda = 6600$ Å.

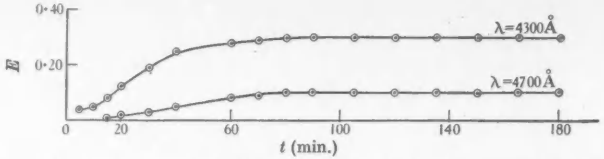


Fig. 2. Series of curves giving the kinetics of extinction for a concentration of methyldichlor-arsine of 0·056 mg./ml. and for various wave-lengths.

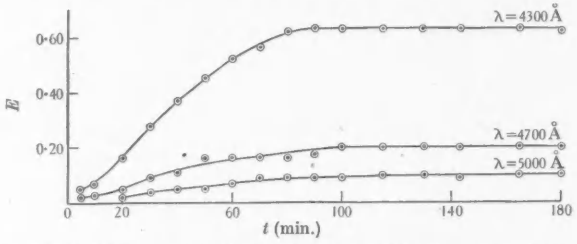


Fig. 3. Series of curves giving the kinetics of extinction for a concentration of methyldichlor-arsine of 0·082 mg./ml. and for various wave-lengths.

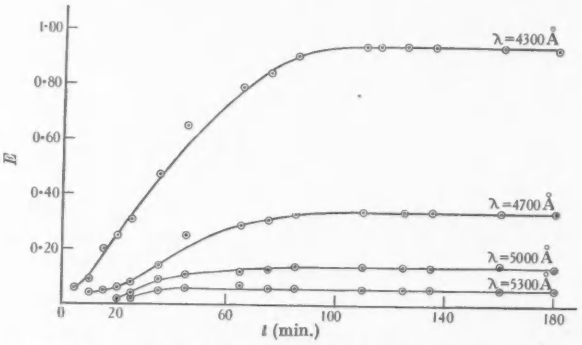


Fig. 4. Series of curves giving the kinetics of extinction for a concentration of methyldichlor-arsine of 0·107 mg./ml. and for various wave-lengths.

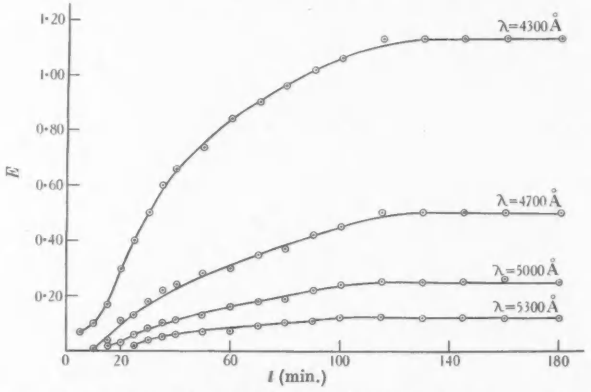


Fig. 5. Series of curves giving the kinetics of extinction for a concentration of methyldichlor-arsine of 0·122 mg./ml. and for various wave-lengths.

Methods

The following precautions are absolutely necessary: (1) The substances used must be strictly dry and pure.* (2) The chloroform solutions of ergosterol must be freshly prepared.

For each concentration of methyldichlor-arsine utilized a known quantity of the compound is added to 5 ml. of a 3 % chloroform solution of ergosterol. This mixture is poured into a cell 10 mm. thick and this latter placed in the heated space of the Pulfrich photometer, the temperature of which is maintained constant throughout the experiment.

The development of the tint is observed by determining the extinction coefficients E, as a function of the time t, for the following wave-lengths: 4300, 4700, 5000, 6100, 6600, 7200, 7500 Å. All these measurements were made by comparison with a 10 mm. cell containing pure chloroform.

Results

The complete kinetic study was made at 46–47° and the results obtained are shown in a condensed form in Figs. 2–5.

It is to be noted that for a given concentration of arsine the extinction coefficients reach a maximum value, E_M, and that, within the range of concentrations utilized

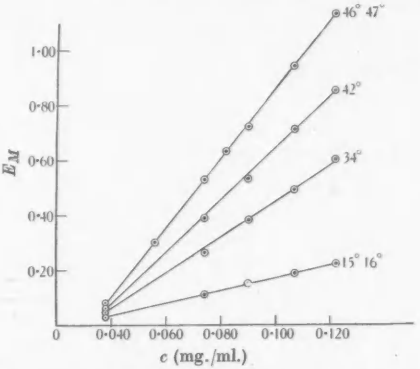


Fig. 6. Extinction maxima as functions of concentrations at various temperatures.

(0·040–0·122 mg.), the values of these maxima E_M are a linear function of the concentrations of arsine introduced at the start of the reaction; at lower temperatures, the maximum extinction coefficient is obtained at the end of a longer period of time (Fig. 6). Hence a method of titration could be obtained for methyldichlor-arsine.

SUMMARY

Ergosterol in chloroform solution gives, in the presence of traces of methyldichlor-arsine, a golden yellow tint, the intensity of which reaches a maximum value after a certain time.

The maximum extinction coefficient (E_M) and the corresponding concentration of the arsine (c) are related by the equation $E_M = kc$ (Beer-Lambert's Law).

* The ergosterol (non-irradiated) was supplied by Messrs Byla and Co. The methyldichlor-arsine was a pure product, b.p. 132·5°, and gave on analysis:

	Found	Calc.
Cl %	43·89	44·09
As %	46·72	46·50

The chloroform was rectified several times and carefully dried.

82. The Effect of Salicylate and Cinchophen on Enzymes and Metabolic Processes

By **Cecilia Lutwak-Mann**,* *From the Biochemical Laboratory, Cambridge*

(*Received 30 July 1942*)

The value in rheumatism therapy of salicylic acid was recognized as early as 1876 and cinchophen (phenylcinchoninic acid; atophan) was introduced in gout treatment in 1908. Several of the many clinical observations are of great interest to the biochemist, e.g. the effect of these substances on the excretion of uric acid, their ability to promote the secretion of bile etc. More recent chemical studies were mainly concerned with attempts to isolate from urine products of salicylate detoxication such as salicyluric acid and salicylglucuronide. Chemical work on cinchophen dates back to the time of Skorczewski & Sohn [1911] and Dohrn [1912], who isolated various oxidation products of cinchophen from human urine.

However, information is far from complete with regard to the actual mechanism in which salicylate and cinchophen exert their beneficial influence on the diseases of the joint. Continuing the work on problems connected with the tissues which make up the joint [Lutwak-Mann, 1940; 1941], a study was made of the effect of salicylate and cinchophen, first on the in vitro activity of isolated tissues and enzyme systems, and secondly on certain metabolic processes in the intact animal (rat).

A. Experiments in vitro

Respiration and anaerobic acid production

Rat liver and kidney cortex, in the form of slices, extracts and pulp, were used in experiments which were carried out at 38° in Barcroft differential manometers for the measurement of the O_2-uptake and the anaerobic acid production. The tissue slices were suspended in Ringer-phosphate *p*H 7·2 for the measurement of respiration and in Ringer-bicarbonate for the CO_2-evolution due to anaerobic acid production. The results recorded below give values (μl./0·2 g. fresh tissue) for the first 20–30 min. when the reactions proceeded at a steady rate; but readings were always continued for another 30–60 min. Salicylate and cinchophen (Na salt), as well as certain benzoic acid derivatives other than salicylic acid, were added in various concentrations to tissue slices respiring either alone or in the presence of certain substrates. Table 1 shows that $M/100$ salicylate had little effect on the O_2-uptake of the liver slices; $M/100$ cinchophen had a slight inhibitory effect which increased markedly at $M/50$ concentration. In $M/20$ salicylate the respiration was well below the control and $M/10$ salicylate caused a considerable inhibition. This effect, however, varied from one animal to another. Table 1 gives also results obtained with $M/10$ benzoate, *o*-aminobenzoate (anthranilate) and *o*-cresotinate. Of these, only *o*-cresotinate inhibited the O_2-uptake to a degree comparable with that of salicylate, while the others had little effect. On the other hand, $M/12$ cinchophen almost completely stopped the respiration of liver slices.

Experiments of this kind were repeated with livers from starved rats and also from rats which received an excess of glucose for one or more days before the experiment, in addition to their ration. With $M/10$ salicylate it was found that the respiration in the starved tissue slices was more affected than either in the normal or the glycogen-rich slices (Table 1).

The addition to the respiring slices of glucose, lactate, dihydroxyacetone or acetaldehyde (all of which were oxidized by the liver slices), did not alter the degree of inhibition

* Fellow of Newnham College, Cambridge.

Table 1. *Effect on the respiration of liver slices*

	O_2-uptake μl. O_2/30 min.		O_2-uptake μl. O_2/30 min.
Control	240	Na cinchophen $M/100$	190
Na salicylate $M/100$	248	Na cinchophen $M/50$	120
Na salicylate $M/60$	198	Na cinchophen $M/12$	36
Na salicylate $M/20$	162	Na benzoate $M/10$	200
Na salicylate $M/10$	120 (normal rat)	Na anthranilate $M/10$	210
Na salicylate $M/10$	99 (starved rat)	Na *o*-cresotinate $M/10$	110
Na salicylate $M/10$	136 (glucose-fed rat)	Na *p*-aminobenzoate $M/10$	268

due to $M/10$ salicylate or $M/20$ cinchophen; when, however, pyruvate was added to the slices, then the inhibition caused by salicylate was somewhat smaller than in the absence of pyruvate.

Kidney cortex slices were on the whole more sensitive than liver to salicylate and cinchophen, but not to benzoate, anthranilate or *p*-aminobenzoate. It may be added that several experiments were carried out with anthranilate and *p*-aminobenzoate to see if they were oxidized and/or deaminated by the rat liver or kidney, with negative results.

Extracts from liver were made by grinding the tissue at 0° with Ringer-Na_2HPO_4 solution (1 part tissue : 2 parts Ringer-phosphate); the residue was removed by centrifugation and the supernatant was used for the experiments. The O_2-uptake of these extracts decreased rapidly after the initial 20–30 min. unless cozymase and nicotinic acid amide were added. Salicylate $M/60$–$M/30$ had relatively little effect, but cinchophen decreased the O_2-uptake at $M/80$–$M/50$ concentration; higher concentrations of salicylate and cinchophen caused a considerable decrease in the O_2-uptake of the extracts. The degree of inhibition was the same whether cozymase and nicotinic acid amide were added or not. The addition of various substrates to the respiring extract made no difference to the inhibitory effect of strong salicylate or cinchophen, except for pyruvate, the presence of which slightly diminished the extent of the salicylate inhibition. The addition of strong methylene blue increased the initial stage of respiration in the liver extracts, and in the presence of this carrier $M/10$ salicylate had a slightly less pronounced effect. When $M/100$ KCN was added to the extract together with methylene blue, then the small 'KCN-resistant' O_2-uptake was completely unaffected by $M/10$ salicylate but was still further depressed by $M/20$ cinchophen. Experiments with liver extracts are given in Table 2.

Table 2. *Effect on the O_2-uptake in liver extract and pulp*

	O_2-uptake μl. O_2/30 min.
Extract (cozymase and nicotinic acid amide added)	
Control	292
Na salicylate $M/30$	200
Na salicylate $M/10$	108
Cinchophen $M/20$	95
Control with 1·5 mg. methylene blue	318
Na salicylate $M/10$ with 1·5 mg. methylene blue	272
Control with 1 mg. methylene blue and KCN $M/100$	78
Na salicylate $M/10$ with 1 mg. methylene blue and KCN $M/100$	70
Na cinchophen $M/20$ with 1 mg. methylene blue and KCN $M/100$	32
Pulp	
Control	174
Na salicylate $M/100$	78
Na salicylate $M/20$	52
Na cinchophen $M/100$	53
Na cinchophen $M/20$	32

A preparation from rat liver which was much more sensitive to salicylate and to cinchophen than either the slices or the extracts, was made by grinding the liver rapidly

(1–2 min., 0°) with one volume of phosphate *p*H 7·4 containing equal molar amounts of K^+ and Na^+. The pulp obtained in this way respired fairly vigorously during the first 30 min. and its O_2-uptake was considerably diminished in the presence of *M*/100 salicylate and still more so, in the presence of *M*/100 cinchophen (Table 2).

Extracts and pulp from rat kidneys behaved towards salicylate and cinchophen like the liver preparations except that their O_2-uptake was lower.

On several occasions Na acetylsalicylate (Na aspirin) was used instead of salicylate. However, with this substance added to either liver or kidney slices or other preparations, the *p*H fell within a short time to less than 5·0. This was found to be due to a very rapid hydrolysis of acetylsalicylate by these tissues. Table 3 gives results of the enzymic hydrolysis of Na aspirin by liver and kidney as well as by other animal tissues and fluids and also by yeast juice.

Table 3. *Enzymic hydrolysis of Na acetylsalicylate (Na aspirin)*

N_2 95 % + CO_2 5 %; $NaHCO_3$ 0·5 %. 38°. Acetylsalicylate *M*/10.

	Acid production μl. CO_2/15 min.			Acid production μl. CO_2/15 min.	
	Control	Acetyl-salicylate		Control	Acetyl-salicylate
Liver slices	22	198	Stomach mucosa	4	36
Liver extract	15	256	Serum	0	12
Kidney cortex slices	18	167	Milk (cow)	0	28
Brain slices	12	88	Yeast juice	28	48

The effect of acetylsalicylate upon the initial stages of the respiration in liver slices was usually less than with a corresponding amount of salicylate; however, in the later stages, when the control respiration began to decrease, the tissue sample containing acetylsalicylate showed a gradual increase in O_2-uptake while the inhibition due to salicylate continued (Table 4). It was thought at first that the phenomenon observed with acetylsalicylate was due to an oxidation of acetic acid, but it was found that acetate added to liver slices was rather poorly oxidized.

Table 4. *Effect of Na salicylate and acetylsalicylate on the O_2-uptake of liver slices and extract*

	O_2-uptake, μl.			
	0–20 min.		50–70 min.	
	Slices	Extract	Slices	Extract
Control	178	120	110	60
Salicylate *M*/10	90	45	45	25
Acetylsalicylate *M*/10	100	77	126	98
Cinchophen *M*/20	78	30	36	15

The anaerobic acid production in liver slices was tested manometrically; it was not affected by *M*/10 salicylate, and with *M*/20 cinchophen there was some inhibition (Table 5). The tissues of starved and glucose-fed animals were also examined, but the results as regards anaerobic acid production were the same as with the tissue of normally fed animals.

Table 5. *Effect of Na-salicylate and cinchophen on the anaerobic acid production in liver slices*

N_2 95 % + CO_2 5 %; 0·5 % $NaHCO_3$.

	Acid production μl. CO_2/30 min.
Control	138
Salicylate *M*/10	126
Cinchophen *M*/20	102

The formation of conjugated glucuronides

It was of considerable interest to test the ability of the rat liver to form in vitro, conjugated glucuronides from salicylate and from cinchophen as well as from certain benzoic acid derivatives other than salicylic acid. Following the technique of Lipschitz & Bueding [1939] for the quantitative estimation of ether-soluble glucuronides, it was first established that rat-liver slices readily formed a glucuronide from *d*-borneol, provided that a very low concentration of this alcohol was used; *l*-menthol was found to be conjugated to a much smaller degree, and occasionally negative results were obtained with menthol, even in the presence of pyruvate, lactate and dihydroxyacetone, all of which, according to Lipschitz & Bueding, enhance the conjugation.

No data exist for the properties of salicylglucuronide, but it may be assumed that it is ether-insoluble like the glucuronide of benzoic acid [Quick, 1926]. Using the method of the authors quoted above, the glucuronic acid estimations were done both in the ether extract from the tissue slices which respired in the presence of the examined substances, as well as in the ether-extracted residue. In later experiments the time-consuming ether extraction was given up and the Tollens test was carried out either (1) directly in the trichloroacetic or tungstic acid extract from the liver slices, or else (2) the protein-free acid extract was brought to *p*H 6·5 and neutral Pb acetate was added avoiding excess; the precipitate was discarded, the supernatant made slightly alkaline with NaOH, and basic Pb acetate was added to complete precipitation; after the removal of Pb from the precipitate, this fraction was used for the estimation of glucuronic acid by means of the Tollens test [Mozołowski, 1940].

Negative results for glucuronic acid were obtained from liver slices which respired in various concentrations of salicylate; in a few experiments, however, with $M/50$ and $M/80$ salicylate, a feeble and unstable positive Tollens test was obtained. Much time was spent in attempting to establish optimum conditions for the conjugation of salicylate, but in the end the conclusion seemed unavoidable that the ability of the rat liver to form a conjugated glucuronide from salicylate is negligible. Negative results were also obtained when using livers from rats which were fed for several days on small amounts of salicylate.

Negative results were also obtained with cinchophen. On the other hand, however, a positive Tollens test was invariably obtained when liver slices respired in the presence of small amounts of benzoate, anthranilate, *m*- and *p*-hydroxybenzoate and *p*-aminobenzoate; however, no satisfactory quantitative measurements could be made by the Tollens test.

Isolated enzyme systems

Indophenol oxidase was prepared from washed and ground heart muscle (pig), and its activity was tested manometrically by measuring the O_2-uptake in presence of *p*-phenylenediamine and cytochrome *c*. The addition of salicylate weaker than $M/20$ had little effect. $M/10$ salicylate caused an appreciable diminution of the initial O_2-uptake, but during the next 10–20 min., when the activity of the control began to decrease, the O_2-uptake gradually rose in the salicylate sample till it reached the control value, and finally, some 40 min. from the onset of the experiment, the O_2-uptake in presence of salicylate was more than twice the control value (Table 6).

With Na cinchophen the indophenol oxidase activity was inhibited at $M/50$ and still more at $M/20$ concentration, both in the early and in the late stage of the reaction (Table 6). It should be added that salicylate and cinchophen were by themselves not oxidized by minced heart or skeletal muscle.

Dehydrogenases: succinic, lactic, malic and α-glycerophosphoric dehydrogenases were tested by means of the Thunberg technique using Latapie-minced and thoroughly washed skeletal muscle as the source of enzymes. With salicylate weaker than $M/20$ little effect was obtained on any of these dehydrogenases; $M/50$ cinchophen caused a pronounced

Table 6. *Effect of Na salicylate and cinchophen on the activity of indophenol oxidase and succinic oxidase*

	O_2-uptake, μl. O_2				O_2-uptake, μl. O_2		
	0–10 min.	10–20 min.	40–50 min.		0–10 min.	10–20 min.	40–50 min.
Indophenol oxidase				Succinic oxidase			
Control	292	198	28	Control	221	144	56
Salicylate $M/10$	132	152	78	Salicylate $M/10$	119	132	99
Cinchophen $M/20$	100	78	20	Cinchophen $M/20$	93	36	17

inhibition, and $M/20$ almost completely prevented the activity especially of the α-glycerophosphoric dehydrogenase (Table 7).

The activity of the succinic oxidase system was also tested manometrically in the presence of cytochrome *c*. The initial O_2-uptake was fairly strongly inhibited by $M/10$ salicylate; in the later stages of the reaction the degree of inhibition diminished, the O_2-uptake gradually increased and in the end it surpassed the control value. No such 'late' effect was observed with cinchophen which inhibited the oxidation of succinate throughout the whole experimental period (Table 6). It is hoped to find an explanation for the effect of salicylate on respiring minced muscle, in the course of future research.

Table 7. *Effect on the activity of muscle dehydrogenases*

1 ml. methylene blue $M/1000$; 38°.

	Time of reduction, min.				
	Control	Salicylate $M/10$	Cinchophen $M/20$	Benzoate $M/10$	Anthranilate $M/10$
No substrate	120	∞	∞	120	120
No substrate + cozymase	45	180	∞	60	48
Lactate + cozymase	3	7	60	3	4
Malate + cozymase	7	13	75	8	10
Succinate	8	19	28	10	10
α-Glycerophosphate	18	60	∞	22	30

Xanthine oxidase activity was tested by means of the Thunberg technique using a preparation from milk [Dixon & Thurlow, 1924]. It was found that $M/10$ salicylate caused considerable inhibition, but a much weaker effect was achieved with equally strong cinchophen; benzoic and anthranilic acid had scarcely any effect, *o*-cresotic acid inhibited the enzyme. Even with $M/100$ salicylate there was a decrease in the xanthine oxidase activity (Table 8). When $M/10$ salicylate was allowed to act on the enzyme preparation for 1 hr. and was then removed by dialysis, the enzyme activity was fully restored. Cinchophen inhibition was also found to be reversible.

Table 8. *Effect on the activity of milk xanthine oxidase*

2 ml. 4% 'casein' preparation; 1 ml. methylene blue $M/1500$; 38°.

	Time of reduction min.		Time of reduction min.
Control	3	Cinchophen $M/10$	12
Salicylate $M/100$	7	Benzoate $M/10$	3
Salicylate $M/60$	9	Anthranilate $M/10$	5
Salicylate $M/10$	32	*o*-Cresotinate $M/10$	29
Cinchophen $M/60$	6		

Several attempts were made to test (manometrically) the oxidation of xanthine in rat-liver slices, but with little success. When, however, liver pulp was used instead of slices, it was possible to obtain a small positive O_2-uptake with xanthine as substrate. Salicylate inhibited the uptake due to xanthine to the same extent as the control respiration.

The dismutation between hexosediphosphate and pyruvate was examined manometrically as described by Green, Needham & Dewan [1937], using a muscle acetone powder or

fresh skeletal muscle extract as the source of enzymes. Cinchophen, $M/50$ to $M/12$, had comparatively little effect, but salicylate decreased the extent of dismutation even at $M/100$ and stopped the reaction at $M/10$ concentration. Benzoate and anthranilate also showed an inhibitory effect but only when used in high concentration (Table 9). When Embden ester was used as substrate for the acid production instead of hexosediphosphate + pyruvate, salicylate seriously inhibited the reaction even in low concentration. The results with salicylate were the same whether muscle acetone powder or an extract from fresh skeletal muscles served as the source of enzymes. Experiments were also carried out using muscle extract prepared from rats injected with salicylate; they are described below (Section B).

Table 9. *The dismutation between hexosediphosphate and pyruvate in presence of salicylate and cinchophen*

Extract from muscle acetone powder. N_2 95% + CO_2 5%; $NaHCO_3$ 0·5%; 38°.

	Acid production μl. CO_2/20 min.		Acid production μl. CO_2/20 min.
Control	192	Cinchophen $M/12$	120
Salicylate $M/100$	87	Benzoate $M/10$	92
Salicylate $M/10$	19	Anthranilate $M/10$	76
Cinchophen $M/50$	172		

Bone phosphatase activity was examined using aqueous-toluene extracts from the bones of young rats. The following substrates were used and their dephosphorylation tested in the presence of salicylate and cinchophen: adenylic acid, inosinic acid, hexosediphosphoric acid, Cori ester, Neuberg ester, Embden ester, β-glycerophosphoric acid. All of them were very rapidly dephosphorylated by the rat bone extract, but the course of the reactions was in no way affected by either salicylate or cinchophen. Similar results were obtained by Lutwak-Mann [1940] with calf articular cartilage as the source of phosphatase. Experiments with phosphatase from the bones of rats injected with salicylate are described below (Section B).

Other enzymes examined and found not to be much affected by salicylate and cinchophen were: choline esterase (serum), tyramine oxidase (liver), methylglyoxalase (liver), adenyl deaminase (muscle), carbonic anhydrase (laked blood corpuscles). Two plant enzymes, the horse-radish peroxidase and catechol oxidase from mushrooms, were found to be resistant to both salicylate and cinchophen. Enzymes in this group were also tested in the presence of benzoate, anthranilate, *p*-aminobenzoate, *o*-cresotinate, *m*- and *p*-hydroxybenzoate, but their activity was not influenced by these substances.

Yeast enzymes. Some experiments were made with fresh bakers' yeast, dried brewers' yeast and a purified carboxylase preparation from yeast-juice [Green, Herbert & Subrahmanyan, 1941]. The fermentation and oxidation of glucose by fresh bakers' yeast was found to be only very slightly inhibited by $M/10$ salicylate when the reaction was carried out at *p*H 7·0–7·2. But at *p*H 6·5 both processes were quite considerably inhibited by $M/10$ salicylate (Table 10). Weaker salicylate had little effect regardless of *p*H. Acetylsalicylate was deacetylated by fresh yeast as well as by yeast extracts. $M/10$ cinchophen

Table 10. *Oxidation and fermentation of glucose by bakers' yeast in presence of salicylate and cinchophen*

	O_2-uptake μl. O_2/20 min.		CO_2-production in N_2 μl. CO_2/20 min.	
	*p*H 6·5	*p*H 7	*p*H 6·5	*p*H 7
Control	258	298	297	212
Salicylate $M/10$	94	212	106	190
Cinchophen $M/10$	12	28	0	0

completely prevented the fermentation and oxidation of glucose by bakers' yeast (Table 10), and weaker solutions caused a large inhibition. The effect of both salicylate and cinchophen on fresh yeast was reversible: when they were removed from the yeast by washing, its activity was found to be normal.

Decarboxylation of pyruvate was found to be considerably inhibited by $M/10$ salicylate and cinchophen; benzoate and *o*-aminobenzoate, $M/10$, also depressed the reaction though to a smaller extent. Low concentration of salicylate still caused a slight inhibition (Table 11). The results with carboxylase and salicylate were the same, using as the source of enzyme either dried brewers' yeast, cell-free juice from bakers' yeast or a purified enzyme preparation. In the presence of citrate instead of phosphate buffer the inhibitory effect was somewhat less. When the rates of glucose fermentation and pyruvate decarboxylation were compared using the same dried brewers' yeast, it was found that carboxylase activity was more affected by $M/10$ salicylate than the CO_2 production from glucose.

Table 11. *Effect on the activity of yeast carboxylase*

Carboxylase preparation from bakers' yeast juice, 2 mg. dry weight. 30°.

	CO_2-production μl. CO_2/5 min.		CO_2-production μl. CO_2/5 min.
Control	308	Cinchophen $M/10$	172
Salicylate $M/100$	248	Benzoate $M/10$	256
Salicylate $M/10$	38	Anthranilate $M/10$	222

B. Experiments on the whole animal

Young male rats (150–250 g.) received subcutaneous injections of salicylate, Na cinchophen and also of benzoic acid derivatives other than salicylate, in doses varying from 1·0 to 0·1 mg./g. body weight. The following observations were made concerning the behaviour of the injected animals. Salicylate: the dose 1·0 mg./g. was tolerated only if the animals were given an excess of glucose (5–10 % solution substituted for water) in addition to their normal diet during one or more days preceding the experiment; otherwise this dose caused a 50 % mortality. Although there were individual variations in the response of the rats to large amounts of salicylate, yet their behaviour after the injection was very typical. About 4 hr. after the injection, but seldom before, the rats showed various degrees of distress, from drowsiness and muscular limpness to vomiting, muscular twitches and severe convulsions. The bladder sphincter was frequently paralysed; this resulted in a continuous drip of urine and a brown staining of the moist abdominal coat (vide infra). This was the most critical period and in fatal cases death occurred at that time. The glucose-fed animals survived this stage but showed little improvement in the next few hours. Usually from the 7th hr. onwards the condition gradually improved and 24 hr. later most animals appeared fully recovered. Occasionally the recovery was delayed and did not take place till 30–40 hr. after the injection.

Doses of salicylate below 1·0 mg./g. were comfortably tolerated. Among the benzoic acids used for injection experiments, *o*-cresotinate was found to be too toxic when injected (1·0 mg./g.) and invariably caused death from convulsions within 1 or 2 hr.; *p*-cresotinate was decidedly less toxic. Benzoate, anthranilate, *m*- and *p*-hydroxybenzoate, did not affect the animals when injected 1·0 mg./g.

Na cinchophen, 1·0 mg./g., was tolerated by most rats and these showed sleepiness and dragging of hind legs; but there were some animals which died from violent convulsions 4–6 hr. after the injection. In these infrequent cases the urine excreted was almost colourless instead of showing the 'claret' colour typical for urine after well-tolerated cinchophen treatment.

The following routine was adopted for the injection experiments. Several rats of similar weight and age were given extra glucose in addition to their food for a day or two

before the experiment proper. In some experiments glucose was discontinued after the injections, but usually the animals had 3% glucose as their drink in addition to their usual food. The injected animals were placed in metabolism cages for urine collection. At 4, 7 and 24 hr. intervals after the injection the animals were killed and various organs were removed for analysis.

Feeding, instead of injections, of salicylate and cinchophen was attempted but was abandoned because the animals refused food and drink which contained more than 20–40 mg. per day of these substances. On the other hand, they did not object to the addition in their food of benzoate or anthranilate up to 0·3 g. per day.

The enzymic activity of tissues after injections

(*a*) *The respiration of liver slices* was measured manometrically at various intervals after the injection of salicylate and compared with the activity of liver slices from untreated animals. No striking changes were observed with doses ranging from 0·1 to 1·0 mg./g., regardless of whether the O_2-uptake was measured in the presence or absence of glucose, lactate, dihydroxyacetone or xanthine. In some of these experiments, however, a small increase in respiration was noticeable in the liver slices obtained 4 hr. after the injection of salicylate; inhibition was not observed at any stage. A similar slight rise was occasionally registered 4 hr. after the injection of Na cinchophen (1·0 mg./g.). Otherwise there were no changes in the O_2-uptake even in the livers of very distressed animals. Table 12 gives the results of these experiments. Benzoate 1·0 mg./g. had no effect at 4 hr., but in some experiments there was a small increase in the respiration 7 hr. after the injection.

Table 12. *O_2-uptake in liver slices from rats injected with salicylate and cinchophen (1 mg./g.)*

μl. O_2/20 min.

Substrate	Untreated rat	Rats injected: Time after injection hr.	With salicylate	With cinchophen
None	211	4	239	222
		7	220	258
		24	218	220
Lactate	240	4	278	256
		7	246	260
		24	237	230
Dihydroxyacetone	312	4	320	
		7	325	
		24	310	
Glucose	300	4	336	
		7	316	
		24	293	

Several experiments of this kind were done with rats maintained on a B_1-deficient diet (in the early stage of deficiency). The control liver slices of these rats oxidized dihydroxyacetone rather poorly in contrast to normal animals; after the injection of salicylate the liver slices failed altogether to oxidize dihydroxyacetone. The slight increase in respiration 4 hr. after salicylate injection was noticeable in most B_1-deficient rat livers; in addition there was often a similar rise in the livers from rats killed 24 hr. after the injection.

(*b*) *The dismutation of hexosediphosphate and pyruvate.* Extracts were made from the skeletal muscles, 4, 7 and 24 hr. after the injections and their activity compared with that of extracts from untreated animals. These experiments were repeated several times but no difference was found between the activity of the 'salicylate' and the 'control' muscle extracts.

(c) *The dephosphorylation of various substances by bone extract.* Four young rats (90 g.) were injected with salicylate (1·0 mg./g.) and killed 6 hr. later, when their bones were removed and an aqueous extract was prepared from them; the bone extract from four litter mates served as control. The bone phosphatase from the salicylate-treated rats was slightly but distinctly less active than the control towards adenylic acid, hexosediphosphoric acid, Neuberg ester and β-glycerophosphoric acid, but not towards Cori ester or inosinic acid.

In other experiments older animals were used and the activity of the bone extract was examined at 4, 7 and 24 hr. intervals after salicylate injection; however, in these experiments the phosphatase activity showed no change as compared with the control.

Liver glycogen

The measurement of respiration in liver slices after the injections was accompanied by estimations of liver glycogen by Pflüger's method (2–3 g. tissue). To obtain control values for liver glycogen an untreated rat's liver was analysed at the onset of the experiment while a second control animal was killed simultaneously with the last (24 hr.) injected rat. While the respiration of liver slices after salicylate and other substances differed but slightly from the control, liver glycogen showed striking behaviour in response to the various substances examined.

Salicylate caused an almost complete disappearance of glycogen from the liver 4–7 hr. after the injections; but 24 hr. later the glycogen was found to be fully restored to control level and moreover, in many experiments, it surpassed the control values by 30–50 %. Results such as these were best obtained with full doses of salicylate, but they were also observed with 0·5 and 0·2 mg./g. and, also, regardless of whether glucose was fed or discontinued during the actual experiment. There were, however, animals the livers of which had low glycogen values 24 hr. after the injection of salicylate; this happened invariably in rats which continued to show signs of distress for more than 24 hr. after the injection (Table 13).

Owing to the fairly high mortality rate it was not practicable to carry out salicylate injection experiments on starving animals, but data were collected for rats which did not receive additional glucose before the experiment. Here, the 4 hr. values for glycogen were low and they did not improve 24 hr. later (Table 13).

Table 13. *Behaviour of liver glycogen after injection of salicylate and cinchophen. Normal and B_1-deficient rats*

			% glycogen in liver			
			Injected rats, time after injection			
	Exp. no.	Dose injected per g. body wt.	4 hr.	7 hr.	24 hr.	Untreated rats*
		Normal rats				
Glucose added to diet	1	—				2·98
		1 mg. salicylate	0·38	0·11	4·42	
	2	—				3·82
		1 mg. salicylate	0·08	0·04	1·22	
	3	—				3·86
		0·3 mg. salicylate	0·19	0·46	5·80	
No glucose added	4	—				1·52
		0·8 mg. salicylate	0·12	0·26	0·66	
		B_1-deficient rats				
Glucose added to diet	5	—				2·12
		0·7 mg. salicylate	0·10	0·26	0·98	
		Normal rats				
Glucose added to diet	6	—				4·92
		0·8 mg. Na cinchophen	0·92	1·58	3·33	

* Killed 24 hr. after the beginning of the experiment.

B_1-deficient rats which prior to the experiment consumed large amounts of glucose, showed a nearly complete disappearance of liver glycogen soon after the salicylate injection, and although glucose was given all the time, not much more glycogen was found in the livers at the 24 hr. interval (Table 13).*

The behaviour of liver glycogen after injection of 1·0 mg./g. of Na cinchophen was as follows: a fall in the glycogen content after 4 hr. but seldom as drastic as after salicylate, no change or a slight increase after 7 hr. and a return to almost control level 24 hr. later. Never was the control level at this stage exceeded by the glycogen content of the cinchophen-treated animal. In a few cases cinchophen caused a substantial loss of liver glycogen soon after the injection and the corresponding urine sample contained glucose (see later).

To compare the effect of salicylate with that of other benzoic acids, experiments were carried out with aspirin, benzoate, anthranilate, *o*-cresotinate and *m*-hydroxybenzoate. Acetylsalicylate, 0·8 and 0·2 mg./g., was injected to rats fed on glucose; the injection was well tolerated. The loss of glycogen after the larger dose was slow and reached its maximum 24 hr. after the injection; the smaller dose resulted in the disappearance of liver glycogen 7 hr. after the injection, but on the next day there was some increase (Table 14). *o*-Cresotinate, owing to its toxicity, could only be injected in small doses (0·6 mg./g.); very little glycogen was found in the livers during 24 hr. after the injections. After 1·0 mg./g. of anthranilate comparatively little glycogen was lost from the liver after 4 hr., more disappeared during the next few hours, but never as much as after a similar dose of salicylate; on the next day the glycogen was largely, but not fully, restored to control level. With *m*-hydroxybenzoate there was a gradual and progressive loss of glycogen throughout the 24 hr. period. Benzoate caused a fall in glycogen up to 7 hr. after the injection; 24 hr. later there was an increase, but the values were still below the control (Table 14).

Table 14. *The effect of various derivatives of benzoic acid on liver glycogen*

Exp. no.	Dose injected per g. body wt.		% glycogen in liver: Injected rats, time after injection 4 hr.	7 hr.	24 hr.	Untreated rats
1	Acetylsalicylate	0·8 mg.	1·77	1·40	0·35	2·70
		0·3 mg.	0·76	0·09	0·68	
2	Benzoate	1 mg.	1·23	0·28	2·66	3·85
3	Anthranilate	1 mg.	3·00	0·82	3·12	5·00
4	*o*-Cresotinate	0·6 mg.	0·72	0·04	0·27	3·70
5	*m*-Hydroxybenzoate	0·8 mg.	1·40	0·92	0·63	5·00

At this stage the behaviour of glycogen in the presence of salicylate was examined in liver slices. However, no significant changes were obtained with the slices when glycogen and ferricyanide-reducing sugar were estimated at various intervals of incubation with different concentrations of salicylate.

Blood

The above-described behaviour of liver glycogen in the rat led one to expect changes in the *blood-sugar* level. Estimations were carried out, using the method of Hagedorn & Jensen [1923], and the results of one of these experiments are in Table 15. No marked changes were found at any time after the injection of salicylate which would reflect those

* The KOH-hydrolysates from B_1-deficient rat livers frequently gave a green-coloured glycogen precipitate on addition of alcohol. This was not due to salicylate treatment, as it occurred also in the livers of the untreated deficient animals.

Table 15. *Glucose and lactic acid content in blood after injection of salicylate (0·8 mg./g.)*

		mg. % glucose normal rats	mg. % lactic acid: Normal rats	mg. % lactic acid: B_1-deficient rats
Untreated rats		115	20	25
Rats injected with salicylate				
Time after injection	4 hr.	110	18	27
	7 hr.	124	18	22
	24 hr.	120	23	21

which occurred under such conditions in the liver glycogen. *Lactic acid* was estimated by the method of Friedemann, Cotonio & Shaffer [1927]; no striking differences were found either in normal or in B_1-deficient rats in response to salicylate (Table 15). *Pyruvate* was estimated by the method of Bueding & Wortis [1940] in rats injected with acetylsalicylate. The 4 and 7 hr. values in the injected animals were lower than the control blood pyruvate, but the 24 hr. values were equal to the control. The colorimetric estimation of the 2:4-dinitrophenylhydrazone was made inaccurate by the appearance of a yellowish colour in the acetylsalicylate samples. *Blood phenol* estimations were carried out after the injections of salicylate, benzoate and cinchophen using the method of Theis & Benedict [1924] but were rendered difficult owing to the small amount of blood available from single rats. It was, however, possible to observe a considerable rise in the phenol values in blood, which persisted beyond 24 hr. after benzoate and salicylate injection. No increase was seen after cinchophen treatment.

Urine

General properties. Samples of urine from injected and untreated rats were collected for analysis at the 4, 7 and 24 hr. intervals. The volume, *p*H, colour and presence of albumen in the urine were recorded. Salicylate in doses 1·0–0·8 mg./g. invariably caused the excretion of large amounts of pale, almost colourless urine; polyuria was sometimes observed also after much smaller doses, but the colour remained normal. The increased urine excretion continued for a day or two after the injection. The 0–4 hr. samples after large doses of salicylate were alkaline, *p*H 7·6–8·0, and were found to contain bicarbonate. Small doses did not cause bicarbonate excretion. Between 4 and 7 hr. a much more acid urine was excreted irrespective of the dose, and from then onwards the *p*H varied between 6·5 and 6·9. Salicylate always caused albuminuria regardless of the amount injected; albumen was present for several days after a big dose. The test for salicylic acid with $FeCl_3$ was positive after the injection from the earliest moment. It was still positive 5–6 days following a large dose and 2–3 days after smaller doses.

Cinchophen injections were followed by the excretion for 2–3 days of 'claret'-coloured urine, there were no changes in the *p*H, and only sometimes the volume was increased. Albuminuria was rare. The urine gave the following reactions: it turned yellow on addition of conc. HCl, gave a precipitate with phosphotungstic acid, and turned green on addition of ammonium sulphate + NH_3.

Benzoate (1·0 mg./g.) caused the excretion of copious urine of normal colour; early samples were alkaline and contained some bicarbonate; there was no albuminuria. Cresotinates, *o*- and *p*-, were followed by polyuria and very slight albuminuria. There were no changes in volume, colour or *p*H after *p*- and *m*-hydroxybenzoate. Urine excreted after *p*-aminobenzoate showed a distinct yellow colour in the early samples.

Urine excreted after the injection of anthranilate was mostly of normal colour; the *p*H of the early samples varied from 7·0 to 7·8. On standing, this urine sooner or later turned red-brown. The colour first developed on the air-exposed surface and gradually pervaded the whole sample; this change occurred also below *p*H 7·0 though not very readily.

Only the early samples after anthranilate injection showed this spontaneous darkening in contact with air. The length of excretion of anthranilate itself was followed by observing the fluorescence it gave in ultraviolet light. Its presence in urine could be detected in this manner several days after the injection of 1·0 mg./g.

Reduction of Benedict's reagent and ammoniacal Ag. Behaviour towards alkalis. The behaviour of liver glycogen after the injection of salicylate and cinchophen and the other substances used in these experiments, suggested the possibility of sugar excretion in urine. Accordingly, all urines were tested using Benedict's qualitative reagent, and the following observations were made. Positive reduction results were obtained after large doses of salicylate in the 0–7 hr. samples; 7–24 hr. urine showed only a trace of reduction, and later samples did not reduce the reagent at all. Polarimetric readings in strongly reducing samples revealed dextrorotation. After the incubation of such urine with fresh bakers' yeast at *p*H 6·5 the reduction was very largely diminished, but it did not disappear completely (Table 16). Only in one case was laevorotation recorded in salicylate urine; in this sample the reduction after fermentation with yeast remained unchanged. Reduction results in urine excreted after small doses of salicylate are also given in Table 16.

Cinchophen urine very seldom reduced Benedict's reagent. In a few experiments there was reduction in the early sample. The sugar, presumably glucose, showed dextrorotation and was fermentable. The excretion of urine such as this always coincided with a serious loss of liver glycogen.

Early urine samples after the injection of benzoate, *m*- and *p*-hydroxybenzoate, or *o*- and *p*-aminobenzoate, all strongly reduced the copper reagent. All of them showed laevorotation which was particularly conspicuous in the anthranilate urine. The reduction remained unchanged after fermentation with yeast. Doses of *o*-cresotinate of 0·5–0·7 mg./g. caused glycosuria (Table 16). Similar reduction results were obtained both in normal and B_1-deficient rats.

Table 16. *Reduction of Benedict's reagent. Polarimetric readings*

α_D values are for $l = 1$ dm.

Injection	Time of urine excretion: 0–4 hr. Reduction: Before ferment.	0–4 hr. Reduction: After ferment.	0–4 hr. Rotation α_D	4–7 hr. Reduction: Before ferment.	4–7 hr. Reduction: After ferment.	4–7 hr. Rotation α_D	7–24 hr. Reduction: Before ferment.	7–24 hr. Reduction: After ferment.	7–24 hr. Rotation α_D
Salicylate, 1 mg./g.	+++	Trace	+1·09°	+	Trace	0	Trace	Trace	0
Salicylate, 0·1 mg./g.	Trace	Trace	0	+	Trace	0	0	0	0
Cinchophen, 1 mg./g.	+++	0	+0·22°	0	0	0	0	0	0
Benzoate, 1 mg./g.	+++	++	−0·28°	++	++	−0·08°	Trace	Trace	0
Anthranilate, 1 mg./g.	++++	+++	−1·42°	+++	++	−0·78°	+	Trace	Trace
o-Cresotinate, 0·7 mg./g.	++	Trace	+0·08°	+	+		Trace	Trace	0
m-Hydroxybenzoate, 1 mg./g.	+++	+++	−0·29°	++	++		0	0	0
p-Hydroxybenzoate, 1 mg./g.	+++	+++	−0·13°	+	+		0	0	0
p-Aminobenzoate, 1 mg./g.	+++	++	−0·15°	Trace	Trace		0	0	0

When anthranilate urine was added to Benedict's reagent at room temperature the colour changed to a dark blue which gradually deepened to a blackish brown and on further standing Cu_2O was formed. This spontaneous reduction occurred only with early samples, but its extent varied greatly from one experiment to another; it bore no relation to the colour of the urine itself. The test suggested the presence of a strongly reducing substance and, indeed, it was found that this urine was capable of reducing ammoniacal Ag in the cold. It was then noticed that the fluid above the AgO precipitate became pink to purple on standing. Subsequently it was established that all early samples of urine, after the injection of anthranilate, gave a pink to purple colour on addition of NH_3 (or pyridine) in the presence of air. The colour developed and vanished with varying rapidity in

different samples. The test was positive in urine up to 10 hr. after the injection, but some rats excreted the substance responsible for the alkali test for a longer time.

Other urines were then tested both as regards reduction of Ag and the behaviour towards alkalis. It was found that in addition to anthranilate, salicylate, benzoate, *m*-hydroxybenzoate and *o*-cresotinate, all caused the excretion of a strongly Ag-reducing urine. But only salicylate, and to a much smaller extent, *o*-cresotinate, caused the presence in urine of substances which gave a dark colour on addition of alkali. In the presence of NH_3 (not more than 5%) salicylate urine slowly turned a light red-brown colour which was stable for hours. A more typical test was obtained if *N* NaOH was added (0·5 ml./2 ml. of urine) and the sample was allowed to stand without shaking; a dark brown colour began to show on the air-exposed surface within 20–30 min., and after a few hours the whole sample turned dark brown. This colour was stable for days, then it began to fade from the surface downwards, and after a further few days the urine was again almost its original colour. The urine after injection of anthranilate did not give a colour with *N* NaOH, but only with NH_3 or pyridine. Also alkapton urine (obtained by feeding of tyrosine to rats) did not behave towards NaOH in the manner described for salicylate urine. Except for *o*-cresotinate, which was sometimes followed by the excretion of traces of the alkali-susceptible substance, other benzoic acids used in this study, as well as cinchophen, failed to show a positive test with alkalis.

Attempts are being made to isolate and identify the substances responsible for the tests described above. As yet it has been established that they are not precipitated with Hg or Pb acetates and that they are resistant to mild acid hydrolysis at 100°. The dark pigment formed was found to be readily soluble in acid amyl alcohol (but not in ether, benzene or ethyl alcohol), whence it could be brought into aqueous solution by successive washings with dilute Na_2CO_3. The pigment solution showed no absorption band in the visible spectrum; it was not fluorescent. Traces of hyposulphite caused a reversible change of colour from red-brown to yellow, weak H_2O_2 completely destroyed the colour.

When describing the behaviour of the rats after the injection of large doses of salicylate it was mentioned that the abdominal coat, continually moistened with urine owing to incontinence, showed a brown staining. It seems highly probable that this colour was due to the same substance in urine which in vitro turned dark on addition of alkali.

Quantitative estimation of reduction in urine. To follow in a quantitative manner the excretion of the reducing substances in the various urines, use was made of the 2:6-dichlorophenol indophenol titration as applied to ascorbic acid [Birch, Harris & Ray 1933]. The urine was diluted (1 : 4) and acidified with trichloroacetic acid, and the amount of dye per titration was 0·05 ml. (1·0 mg. dye/ml.) which corresponded to 0·02 mg. of ascorbic acid. Care was taken to carry out the titration as soon as enough urine was collected at a given time, but it was found that when an estimation was repeated several hours later, the titration values hardly differed from those obtained at first. It was also found that while with salicylate urine the results were the same whether the titration was carried out in acid or at the *p*H of the urine itself, it was necessary to acidify the anthranilate urine as otherwise very low titration values were obtained.

It is by no means certain that the same reducing substance is excreted in urine after the injection of the various substances studied. The substance in the urine after salicylate injection behaves like ascorbic acid, but the same cannot be said about, for example, cinchophen urine, which is also strongly reducing (see below). However, until the reducing substances are finally isolated and identified it is proposed, for the sake of simplicity, to express the quantitative results obtained as mg. ascorbic acid.

Table 17 gives the results for the reduction in urine in response to various doses of salicylate. It can be seen that all three doses caused a considerable increase in the titratable reduction, but the figures for the whole experimental period (24 hr.) clearly show that there is proportionality between the amount of reducing substances excreted and

the dose injected. In addition, it can be seen that the large dose was followed by polyuria. The high *p*H in the early sample after 1·0 mg./g. salicylate was due to the presence of bicarbonate.

Table 17. *Estimation of reduction in urine by means of 2:6-dichlorophenol indophenol titration, after various doses of salicylate*

icylate jected er g. dy wt.	Time of urine excretion: 0–4 hr. Vol. ml.	*p*H	Ascorbic acid, mg.*	NaOH-test	4–7 hr. Vol. ml.	*p*H	Ascorbic acid, mg.*	NaOH-test	7–24 hr. Vol. ml.	*p*H	Ascorbic acid, mg.*	NaOH-test	Ascorbic acid excreted during 24 hr. mg.
—	0·5	6·0	0·016	–	1·0	6·0	0·019	–	5·5	6·5	0·049	–	0·084
1 mg.	8·0	7·9	1·900	+	4·0	6·7	0·433	+++	16·0	6·5	1·092	++	3·425
6 mg.	6·0	7·7	0·624	+	1·8	6·5	0·240	++	4·5	6·5	0·420	+	1·284
1 mg.	4·0	6·9	0·520	Trace	1·6	6·7	0·110	+	7·0	6·2	0·200	+	0·830

* Total ascorbic acid in volume excreted (also in other Tables).

More experiments of this kind are presented in Table 18. Here, rats were used which did not receive extra glucose, as well as animals which were starved for 20 hr. before the injections. In addition, there are results obtained with B_1-deficient rats fed on glucose before and during the experiment. The data illustrate well the large individual variations in response to the same dose of salicylate, both as regards the reduction and the strength of the alkali test. The deficient animals did not differ essentially from the normal ones, except that the *p*H of the early sample failed to show the rise typical for these samples in normal animals. The same is true of rats which were starved before the experiment.

Table 18. *Reduction in urine after injection of salicylate (0·5 mg./g.) in normal and B_1-deficient rats*

	Time of urine excretion: 0–4 hr. Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	4–7 hr. Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	7–24 hr. Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test
					Normal rats							
dinary diet:												
Not injected	2·0	6·8	0·042	–	1·0	6·8	0·008	–	6·0	6·7	0·102	–
Injected	3·5	7·8	0·123	Trace	2·0	6·9	0·100	++	12·0	6·6	1·062	+
Injected	2·0	7·7	0·224	+	1·0	6·6	0·320	+	8·0	6·4	0·910	Trace
arved 20 hr.:												
Not injected	1·5	6·8	0·060	–	1·5	6·8	0·021	–	7·0	6·6	0·190	–
Injected	4·0	7·0	0·200	Trace	2·0	6·4	0·200	+	10·0	6·6	1·000	+
Injected	4·0	6·8	1·022	+	2·0	6·2	0·321	++	6·0	6·3	0·320	Trace
					B_1-deficient rats							
ucose added to diet:												
Not injected	1·0	5·6	0·011	–	1·0	5·6	0·009	–	4·5	5·5	0·044	–
Injected	2·0	7·0	0·400	+	1·0	6·8	0·110	+	6·5	5·8	0·300	+
Injected	1·5	6·7	0·360	+	1·0	6·8	0·360	++	9·0	6·8	0·210	+
Injected	1·5	6·8	0·170	+	3·0	6·5	0·720	+++	4·5	6·5	0·610	+

Several experiments were carried out injecting rats with benzoate, *m*- and *p*-hydroxybenzoate, *o*- and *p*-aminobenzoate. Table 19 illustrates the results obtained in urine after the injection of 0·65 mg./g. of these substances to rats fed on glucose. A rise in *p*H followed the treatment with benzoate and anthranilate. There was a distinct increase in the reducing power of urine in all, except the urine excreted after the injection of *p*-aminobenzoate. Individual variations as regards reduction were found in response to anthranilate. One may add that the three anthranilate urines differed also in colour, only urines 1 and 2 were brownish; the third one turned brown only after 2 days' standing at 0°.

Table 19. *Reduction in urine after the injection of benzoate, anthranilate, m-hydroxybenzoate, p-hydroxybenzoate, p-aminobenzoate (0·65 mg./g.)*

	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
Injection	Urine ml.	*p*H	Ascorbic acid, mg.	NH_3-test	Urine ml.	*p*H	Ascorbic acid, mg.	NH_3-test	Urine ml.	*p*H	Ascorbic acid, mg.	Ni te
None	1·0	6·0	0·016	–	0·8	6·0	0·019	–	5·5	6·5	0·019	-
Benzoate	3·0	7·5	0·180	–	3·5	6·4	0·350	–	17·0	6·6	0·204	-
Anthranilate 1	2·0	7·6	0·250	++	1·5	6·8	0·150	++	4·0	5·8	0·180	-
Anthranilate 2	1·5	7·5	0·270	+	2·0	6·5	0·684	+	8·0	6·0	0·382	Tra
Anthranilate 3	3·0	7·0	0·362	Trace	1·5	6·5	0·490	+	3·0	6·0	0·150	-
m-Hydroxybenzoate	4·0	6·6	0·640	–	1·0	6·5	0·024	–	3·0	6·7	0·102	-
p-Hydroxybenzoate	6·0	6·7	0·102	–	1·0	6·9	0·020	–	7·5	6·9	0·099	-
p-Aminobenzoate	2·0	7·0	0·056	–	1·0	6·6	0·040	–	2·5	6·6	0·032	-

Results obtained with Na cinchophen as well as with *o*- and *p*-cresotinate are given in Table 20. The large doses of cinchophen and *o*-cresotinate proved too toxic and the animals died 5 hr. after the injections. The smaller dose of cinchophen was followed by the excretion of 'claret'-coloured urine which was strongly reducing not only in the early but also in the later stages of the experiment. Exceptionally high titration values were obtained in this experiment after the injection of 0·6 mg./g. of *o*-cresotinate. *p*-Cresotinate urine also reduced the dye much more strongly than normal urine.

Table 20. *Reduction in urine after the injection of Na cinchophen, o-cresotinate and p-cresotinate*

	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
Dose injected per g. body wt.	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Ascorbic acid, mg.	Na te
None	3·0	6·9	0·095	–	2·0	6·8	0·098	–	9·0	6·7	0·072	-
Cinchophen 1 mg.	6·0	7·1	1·140	–	Rat died							
Cinchophen 0·6 mg.	2·0	6·0	0·420	–	4·0	6·0	0·620	–	22·0	6·3	1·720	-
o-Cresotinate 1 mg.	5·0	6·7	0·475	–	Rat died							
o-Cresotinate 0·6 mg.	4·0	7·0	2·00	Trace	3·0	6·6	0·928	+	28·0	6·4	2·00	Tra
p-Cresotinate 0·8 mg.	2·0	7·0	0·90	–	8·0	6·6	0·442	–	19·0	6·6	0·679	-

While the work on the identification of the reducing substances in the various urines is being continued, it seems worth while to report briefly certain experiments which may help to throw light on the nature of these substances. Two urines were examined, one obtained after the injection of salicylate, the other after anthranilate treatment. To both the procedure of Emmerie & Eekelen [1934] was applied. When the urines were separated with Hg acetate into two fractions, one corresponding to the Hg precipitate, the other to the Hg filtrate, it was found that the filtrate fraction from salicylate urine was invariably more strongly reducing than the insoluble fraction. Exactly the opposite was found in anthranilate urine where the insoluble Hg fraction reduced more strongly than the soluble one.

Other experiments designed to yield more information as to the character of the reducing substances were set up in the following manner. The urine examined (1 ml.) was shaken in air at 20° in Barcroft manometers in the presence of 20 μg. Cu^{++}. Urine without Cu^{++} served as control. A small O_2-uptake was observed in the presence of Cu in salicylate and *o*-cresotinate urine. Table 21 contains the titration results obtained with fresh and Cu-treated urines, expressed in ml. diluted and acidified urine required to reduce 0·05 mg. 2:6-dichlorophenol indophenol. The control and the salicylate urine showed a fall in their reducing power; cinchophen and *o*-cresotinate urine remained unchanged after the exposure to air in presence of Cu.

Table 21. *Effect of air-exposure in presence of Cu^{++} on reducing power of urine*

Ml. urine (acidified and diluted 1:4) required to reduce 0·05 mg. 2:6-dichlorophenol indophenol.

	Before Cu treatment	After Cu treatment		Before Cu treatment	After Cu treatment
Normal urine	2·2	2·9	Cinchophen urine	1·3	1·4
Salicylate urine	1·1	2·2	*o*-Cresotinate urine	0·3	0·36

Behaviour of the alkali test. Data on the behaviour of the salicylate, anthranilate and *o*-cresotinate urines towards NaOH and NH_3 have been included in the tables together with the reduction results. After the injection of salicylate the test was positive from a very early moment regardless of the dose injected. It was still positive 24 and 32 hr. later. On the whole, the test with NaOH appeared in the urine for nearly as long as the $FeCl_3$ test for salicylic acid. In anthranilate urine it was positive only during the first 4–10 hr. and seldom later. After the injection of *o*-cresotinate the test was weakly positive or entirely negative.

An attempt was made to measure manometrically the amount of O_2 taken up by salicylate urine in the presence of *N* NaOH. To 1 ml. urine 0·2, 0·5 and 1 ml. *N* NaOH were added. There was a slow O_2-uptake which continued for 20 hr. at 20° when 72 μl. O_2 were used up by the sample with most NaOH and a little less by the other two. There was no O_2-uptake in the presence of NaOH by normal rat urine.

The behaviour of reducing substances in liver and other tissues. The increased reduction by the urine excreted after the injection of salicylate suggested the possibility of changes in the reducing substances in the tissues of the injected animal. An experiment was carried out using three rats injected with salicylate (1·0 mg./g.) and a control animal, all of whom had glucose before and during the experiment. The livers of these rats were examined at the 4, 7 and 24 hr. intervals for their glycogen content, and the rest of the tissue was used for an estimation of the reducing substances following the procedure of Hopkins & Morgan [1936]. The tissue was ground with sand and trichloroacetic (8%)+ metaphosphoric acid (2%), 20 ml. acid mixture per 10 g. tissue, then water was added to make a 2·5% final acid concentration. One part of the acid protein-free filtrate (corresponding to half the weight of tissue) was titrated with *M*/100 I_2 in the presence of KI till a distinct yellow colour was reached; the end-point was established by means of a back-titration with *M*/100 $Na_2S_2O_3$. The rest of the acid filtrate was used for titration with 2:6-dichlorophenol indophenol in the usual way. The results in Tables 22 and 23 are given as mg. glutathione (GSH) and mg. ascorbic acid per g. tissue wet weight, and in Table 23 also in ml. *M*/100 I_2 per g. tissue. The GSH values were arrived at by converting the ascorbic acid titration into the iodine value and deducting it from the total iodine figure obtained by the titration with *M*/100 I_2 (1 ml. *M*/100 $I_2 \equiv 3{\cdot}07$ mg. GSH).

The results obtained in this way for liver are given in Table 22. In comparison with the control, there was a slight rise in the GSH content 4 and 7 hr. after the injection of salicylate and an almost twofold increase in the GSH content after 24 hr. The level of ascorbic acid remained unchanged throughout the experiment. The liver glycogen showed the usual behaviour, a fall during the first 4–7 hr. and an increase beyond the control value, 24 hr. after the injection.

Table 22. *Estimation of reducing substances and glycogen in liver after salicylate injection (1 mg./g.)*

	Time after injection of salicylate	GSH mg./g.	Ascorbic acid mg./g.	Glycogen g./100 g.	Time after injection of salicylate	GSH mg./g.	Ascorbic acid mg./g.	Glycogen g./100 g.
Control	—	1·20	0·23	3·10	7 hr.	1·53	0·20	0·04
	4 hr.	1·50	0·24	0·12	24 hr.	2·11	0·23	4·90

Several more experiments of this kind were carried out with remarkably similar results as regards the behaviour of the GSH fraction in liver. In other experiments the reducing substances were also determined in the kidneys and in the intestine, not only after salicylate, but also after *o*-cresotinate injection (0·6 mg./g.). The results are given in Table 23. The liver showed the notable rise in the GSH content 24 hr. after both salicylate and *o*-cresotinate. In kidney and intestine there was a distinct rise in the GSH values 4 and 7 hr. after the salicylate injection; the ascorbic acid values at these intervals also exceeded the control, but after 24 hr. there was no further increase and all values were almost completely equal to those found in the untreated rat. The amounts of the reducing substance excreted by each animal are also recorded in Table 23.

Table 23. *Reducing substances in liver, kidney, intestine and urine, after salicylate and* o*-cresotinate injections (0·6 mg./g.)*

	Liver			Kidney			Intestine			Urine
	$M/100\ I_2$ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	$M/100\ I_2$ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	$M/100\ I_2$ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	mg. ascorbic acid excreted during exp. period
Control	0·71	0·30	1·50	0·40	0·19	0·71	0·37	0·20	0·66	0·38/10 ml.
After injection of salicylate										
4 hr.	0·85	0·30	1·77	0·55	0·22	0·88	0·60	0·33	0·98	1·76/16 ml.
6 hr.	6·76	0·22	1·49	0·51	0·23	0·78	0·58	0·35	0·83	3·42/18 ml.
24 hr.	1·10	0·23	2·46	0·44	0·19	0·78	0·36	0·18	0·64	2·8/24 ml.
After injection of *o*-cresotinate										
5 hr.	0·76	0·27	1·32	0·43	0·22	0·85	0·36	0·28	0·74	0·805/7 ml.
24 hr.	1·80	0·32	2·02	0·44	0·23	0·69	0·44	0·29	0·63	2·22/30 ml.

The effect of ascorbic acid on the excretion of the alkali-susceptible substance after the injection of salicylate and on the excretion of homogentisic acid in artificial alkaptonuria

The behaviour of salicylate urine towards alkalis bore a certain resemblance to that of homogentisic acid in alkaptonuria. Since no real alkapton urine was available an attempt was made to induce alkaptonuria artificially by feeding rats with an excess of tyrosine (1–2 g. daily). The experiment was successful, and shortly after the feeding of tyrosine was started the urine gave distinctly positive tests for homogentisic acid (transient blue in acid ether extract with $FeCl_3$, red-brown colour on standing with NH_3, reduction of alkaline copper reagents; in addition, homogentisic acid was identified as dibenzoyl homogentisamide [Papageorge & Lewis, 1938].

The alkapton urine differed from the salicylate urine in that unlike the latter it did not darken in the presence of *N* NaOH but gave a dark colour only with NH_3. Without, however, attaching too much weight to purely qualitative tests, a further difference between these two urines was shown to exist when the effect was studied of treatment with ascorbic acid, (*a*) on the excretion of the 'alkapton'-like substance in salicylate urine, and (*b*) on the excretion of homogentisic acid in alkaptonuria.

Table 24 gives the results of the following experiment: rats (140 g.) were given injections of (1) ascorbate 0·6 mg./g., (2) ascorbate 0·4 mg.+salicylate 0·6 mg./g., and

Table 24. *Effect of ascorbic acid on urine excretion after salicylate injection*

	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
Dose injected per g. body wt.	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Accorbic acid, mg.	NaOH test
None	1·0	6·5	0·068	–	1·0	6·3	0·080	–	4·0	6·3	0·079	–
Ascorbate 0·6 mg.	5·0	5·2	49·0	–	2·0	5·5	5·70	–	8·0	6·3	0·490	–
Ascorbate 0·4 mg. + salicylate 0·6 mg.	3·0	7·0	26·0	Trace	8·0	6·8	2·40	+	30·0	6·9	2·85	++
Salicylate 0·6 mg.	5·0	7·1	1·10	+	11·0	6·6	0·44	+	20·0	6·3	0·60	++

(3) salicylate 0·6 mg./g. Urine collection was started 20 hr. before the injections so as to establish the reducing power of each urine (values ranged from 0·008 to 0·015 mg. ascorbic acid, the volumes from 9 to 11 ml., *p*H was 6·9). Glucose was given only the night before the experiment. It follows clearly from the results presented in Table 24 that in spite of the large dose of ascorbic acid injected, the substance which causes the NaOH test to appear continued to be excreted in response to salicylate. In complete contrast to this all tests for homogentisic acid were negative when ascorbic acid was added to the tyrosine diet of an alkaptonuric rat. The full details of this experiment are given below.

The properties of urine after tyrosine feeding. In addition to the qualitative tests for homogentisic acid, quantitative estimations of reducing power were carried out on the urine of a rat receiving daily 1–2 g. tyrosine with his diet. The titration against 2:6-dichlorophenol indophenol was done in exactly the same manner as with other urines in this study. The results obtained during a 14 days' feeding experiment are given in Table 25. The reduction values are expressed as ml. diluted and acidified urine required to reduce 0·05 mg. dye. The estimations were run twice daily, on the overnight urine and again in the evening.

Traces of alkapton were noticeable already $1\frac{1}{2}$ days after the start of the experiment. During the 3rd day alkaptonuria was fully developed, and in the course of the next few days the qualitative tests became gradually stronger. At the same time, however, the general condition of the rat deteriorated, it developed light-shyness due to keratitis and its paws and tail were remarkably pink. The appetite was not affected. A large amount of glucose solution was substituted for water on the 8th day, but the only effect it had was polyuria, while the tests for alkapton continued positive. On the 11th day the rat received 40 mg. of ascorbic acid in addition to the usual tyrosine dose. The urine tested 14 hr. later gave negative results with $FeCl_3$ and NH_3. The feeding of ascorbic acid was continued for 2 more days, and although during this time the rat consumed 4 g. of tyrosine, there was no alkaptonuria.

The titration results with 2:6-dichlorophenol indophenol were of considerable interest. From the moment when the first traces of alkapton appeared in the urine, the titratable reduction began to rise until it reached a fairly high and steady level. When, however, the large amounts of ascorbic acid were given, there was no further increase in urine reducing power as would be the case with a normal animal. It looked as if the rat retained

Table 25. *Effect of tyrosine feeding on urine excretion in rat. Effect of ascorbic acid on alkaptonuria*

Tyrosine feeding duration	ml. urine (acidified and diluted 1 : 4) to reduce 0·05 mg. indophenol	$FeCl_3$-test	NH_3-test	Reduction of Benedict's reagent
0–18 hr.	3·80	–	–	–
18–24 hr.	3·50	–	–	–
24–48 hr.	3·00	Trace	Trace	Trace
3rd day	1·10	+	+	+
4th day	0·45	+ +	+	+
5th day	0·30	+ + +	+	+ +
6th day	0·60	+ + +	+	+ +
7th day	0·66	+ +	+	+ +
8th day	0·40	+ + +	+	+ +
Rat given glucose (7 g./40 ml.) 9th day	0·72	+ + +	+	+
10th day	0·28	+ + +	+	+
11th day	0·63	+ + +	+	+ +
Rat given ascorbic acid (2 × 20 mg.) 12th day	0·42	–	–	–
13th day	0·80	–	–	–
14th day	0·26	–	–	–

the huge doses of ascorbic acid, perhaps owing to the loss of reducing substance from the body caused by the feeding of tyrosine.

Further interesting results were obtained when an attempt was made to estimate colorimetrically, by the method of Briggs [1922], homogentisic acid in the tyrosine urine, and the alkapton-like substance in salicylate urine. For colorimetric estimation 0·5 ml. samples were used of (*a*) two salicylate urines, and (*b*) tyrosine urine. Their titratable reduction was 0·65 and 1·00 ml. for the salicylate urines, and 0·72 ml. for the tyrosine urine (diluted and acidified), against 0·05 mg. dye. To all three samples 2 ml. molybdate and 2 ml. KH_2PO_4 were added; the final volume was made up with water to 25 ml. The standard solution containing hydroquinone (1 mg./ml.) was treated in the same manner and standards ranging from 0·2–20 mg./25 ml. hydroquinone were prepared (1 mg. corresponds to 0·79 mg. homogentisic acid). The blue-green colour which developed in a few min. in the two salicylate urines matched 0·2 and 0·4 mg. hydroquinone/25 ml. The tyrosine urine, however, on addition of molybdate turned a deep yellow which quickly changed into olive green, and when phosphate was added a dark blue colour was obtained which exceeded in intensity 20 mg. hydroquinone/25 ml. It must be pointed out that the strength of the qualitative tests for homogentisic acid in this urine was not such as to suggest a very high content of homogentisic acid, and it seems probable that yet another reducing substance in this urine was responsible for the results obtained with molybdate and phosphate.

To gain information about the character of the reducing substance in tyrosine urine as compared with salicylate urine and also with urine which contained added ascorbic acid, an experiment was set up similar to that described above. The following urine samples were titrated before and after exposure to air, in the presence of Cu-salicylate urine, alkapton urine, urine from a rat which was fed excess of ascorbic acid, and normal rat urine to which ascorbic acid had been added. Table 26 gives the titration results expressed as ml. diluted and acidified urine required to reduce 0·05 mg. dye. All urines, except the alkapton urine, showed a substantial fall in the reducing power after the treatment with Cu.

Table 26. *Effect of air-exposure in presence of Cu^{++} on reducing power of urine*

1 ml. urine + 30 μg. Cu^{++}; 2 hr.; air; 18°.

Sample	ml. urine (acidified and diluted 1 : 4) to reduce 0·05 mg. indophenol	
	Before treatment	After treatment
Salicylate urine	1·00	2·80
'Alkapton' urine	0·30	0·50
Urine after feeding excess ascorbic acid	1·00	3·20
Normal urine + added ascorbic acid	0·42	2·20

Experiments with salicylic aldehyde

It was shown by Dixon & Lutwak-Mann [1937] that liver contains two enzymes capable of converting in vitro an aldehyde into an acid; the aldehyde oxidase which was found to act equally well on aliphatic and aromatic aldehydes, and the mutase which acted readily on aliphatic but only very poorly on aromatic aldehydes. It was interesting to examine the effect in vivo of salicylic aldehyde and to compare the results obtained with those established for salicylic acid.

In the preliminary experiment two rats fed on glucose were given 0·8 mg./g. of salicylic aldehyde (aqueous emulsion *p*H 7·0). In spite of the comparatively large dose there were no signs of distress, and the animals looked decidedly better than after a similar dose of salicylate. Table 27 contains the results on urine excretion. The earliest sample was alkaline and of yellow colour (due to the presence of salicylic aldehyde which turns yellow

above *p*H 7·0), there was no albuminuria, no increase in the reducing power, and the test with *N* NaOH was negative in one rat but weakly positive in the other. However, the urine excreted later showed an increase in reduction and a fully developed alkali test in both rats. There was a further rise in reduction in the 10–24 hr. urine samples, but the test with NaOH was weaker. Albumen was present only in the later samples.

Table 27. *Urine excretion after the injection of salicylic aldehyde (0·8 mg./g.)*

	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
Injection	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Urine ml.	*p*H	Ascorbic acid, mg.	NaOH test
None	2·5	6·9	0·069	–	0·5	7·0	0·085	–	2·0	7·0	0·092	–
Salicylic aldehyde 1	4·0	8·0	0·092	–	3·0	6·9	0·300	++	15·0	6·9	0·750	++
Salicylic aldehyde 2	4·0	8·0	0·098	Trace	4·0	7·0	0·600	+++	17·0	6·9	1·360	++

In another experiment three rats received salicylic aldehyde (0·8 mg./g.) and three others salicylate (0·8 mg./g.), while two of the same group were kept as controls. All had extra glucose before the start of the experiment. Liver glycogen was estimated 4, 7 and 24 hr. after the injections. Table 28 gives the results obtained for liver glycogen and urine excretion. It illustrates the difference in the response of the rat towards salicylate and salicylic aldehyde as regards the reducing power of urine, albuminuria and the strength of the NaOH test. The usual results were obtained for liver glycogen after the injections of salicylate, i.e. exhaustion of glycogen in the 4–7 hr. period followed by recovery on the next day. The change due to the aldehyde after 4–7 hr. was less pronounced, but the recovery after 24 hr. was almost as good as after salicylate itself.

Table 28. *Comparison between the effect of salicylate and salicylic aldehyde (0·8 mg./g.) on urine excretion and on liver glycogen*

		Time of urine excretion hr.	Urine				Liver glycogen %
Exp.	Substance injected		Vol. ml.	*p*H	Ascorbic acid, mg.	NaOH-test	Albumen	
1	Salicylate	0–4	5·0	8·0	1·120	+	+	0·40
	Salicylic aldehyde	0–4	2·0	8·0	0·035	Trace	–	1·60
2	Salicylate	0–7	8·0	7·8	1·760	+	+	0·80
	Salicylic aldehyde	0–7	4·0	7·0	0·320	+	–	0·80
3	None	0						5·00
	None	0–3½	2·0	6·9	0·092	–	–	
	None	3½–18	5·0	5·9	0·109	–	–	
	None	18–24	3·0	6·9	0·088	–	–	4·10
	Salicylate	0–3½	5·0	7·9	0·650	+	+	
	Salicylate	3½–18	12·0	6·8	1·20	++	+	
	Salicylate	18–24	4·0	5·2	0·160	++	+	4·70
	Salicylic aldehyde	0–3½	6·0	8·0	0·130	+	–	
	Salicylic aldehyde	3½–18	12·0	5·7	1·400	++	Trace	
	Salicylic aldehyde	18–24	2·0	5·8	0·210	Trace	Trace	3·90

Discussion

The object of this work was to gain information about the mechanism of action of salicylate and phenylcinchoninate. To this end two types of experiments were carried out: (*a*) on tissues separated from the body and on isolated and partially purified enzyme systems, and (*b*) on the whole animal, by introducing these substances parenterally and studying their effect at intervals chosen so as to coincide with marked changes in the behaviour of the injected animals. On the whole, there is not unsatisfactory agreement in the results obtained by means of these two experimental methods. For instance, it was found in experiments on liver slices in vitro that only relatively high concentrations of salicylate were able to depress the respiration. Correspondingly, the respiration of

liver slices from injected animals was found not to be much affected even after a relatively high dose of salicylate. The inability of rat liver to form in vitro salicylglucuronide was reflected in the findings on urine excretion, which proved the absence of appreciable amounts of glucuronide in response to salicylate treatment. On the other hand, glucuronides were found to be excreted after, for example, benzoate or *o*-aminobenzoate, both of which gave rise to glucuronide formation in vitro. With regard to the effect of salicylate on the dismutation between hexosediphosphate and pyruvate, one would have expected from in vitro results this reaction to be more sensitive to the injection of salicylate than was actually found.

The proof that profound changes in metabolism are provoked by the injection of salicylate and cinchophen, as well as by some of the benzoic acid derivatives examined, is provided by the results obtained with liver glycogen and the changes in the composition of reducing substances in the tissues. The disappearance soon after the salicylate injection of nearly all the liver glycogen and its full restoration 24 hr. later, clearly indicate an effect on several as yet unidentified enzymic processes. The differences in the behaviour of liver glycogen in response to the various substances used in this work suggest that the fall in glycogen may be brought about in a different way in every case. In B_1-deficient rats, while the results for urine excretion differed but little from those obtained with normal rats, there were differences in the behaviour of liver glycogen, and also it was found that the oxidation of dihydroxyacetone by liver slices of the deficient animals was diminished by salicylate.

The study of urine excretion brought out several facts as regards the effect on the animal organism of salicylate in comparison with other derivatives of benzoic acid. Certain features, such as the increase in the titratable reducing power of urine, were found common to most of them, but bicarbonate was excreted only after salicylate, benzoate and anthranilate. Albuminuria was typical after salicylic acid, irrespective of the dose. Yet more specific were the tests obtained in the presence of alkalis: only ortho-substituted acids such as salicylic, anthranilic and *o*-cresotic, caused the excretion of substances which formed dark pigments with alkalis in the presence of O_2. The specificity of salicylate was further underlined by the findings with salicylic aldehyde. It was obvious that the aldehyde itself did not cause an increase in the reducing power of the urine, or albuminuria. As soon, however, as some of it had been transformed in the body into the acid, a matter of a few hours in the rat, the urine became strongly reducing, contained albumen, and showed a strongly positive test with NaOH.

The injection of Na cinchophen also increased greatly the titratable reducing power of urine, which, however, was not affected in the least by exposure to air in the presence of catalytic amounts of Cu, as was the substance responsible for the increased reduction in salicylate urine. Resistance of the reducing substance to oxidation was also shown by urine excreted after the injection of *o*-cresotinate and after feeding with tyrosine. On the other hand, ascorbic acid added to normal urine was sensitive to air exposure in the same manner as salicylate urine. This similarity suggests, but by no means proves, that ascorbic acid may be the substance excreted by the rat in response to salicylate treatment.

There is no doubt that the reducing substances estimated by titration in the salicylate and anthranilate urines are not identical with those which give dark-coloured pigments with alkalis. This is borne out by results in which the alkali test was strongly positive and the reduction slight, e.g. samples excreted late after the injection of salicylate; vice versa, there were strongly reducing urines with a very weak alkali test, like the *o*-cresotinate urine. While it remains for future work to identify the substances responsible for the behaviour towards alkalis, one can claim with some certainty based on the striking difference in the behaviour towards ascorbic acid treatment, that the alkapton-like substance which appears in urine during salicylate treatment is not identical with homogentisic acid.

The findings made with regard to reducing substances in the urine and in the tissues (particularly liver) are of interest in connexion with the observations of Abbassy, Gray-Hill & Harris [1936], who stated that in acute rheumatism as well as in rheumatic arthritis the excretion of ascorbic acid in response to large doses of it is much smaller than in normal subjects. Possibly in rheumatic disease it is the metabolism of the reducing substances which is affected in some hitherto unknown way, and the beneficial influence of the anti-rheumatic drugs may be linked with their ability to influence this phase of metabolism.

There are other recent indications that a strongly reducing substance, such as ascorbic acid, may be involved in the pathology of the joint tissues. It has been shown by Robertson, Ropes & Bauer [1941; 1942] that the mucin of synovial fluid, as well as collagen from tendons, undergo far-reaching changes in the presence of ascorbic acid and small amounts of H_2O_2. It is hoped that further research on the anti-rheumatic drugs will help to bring into a logical whole the accumulating facts and observations, and thus accelerate the solution of the clinical problems of rheumatic disease.

Summary

The effect of salicylate and cinchophen was investigated on isolated enzyme systems and in the whole animal. Normal as well as B_1-deficient rats were used. The action of salicylate was compared with that of other benzoic acid derivatives.

The respiration of slices and extracts from rat liver and kidney cortex was not much affected by salicylate up to $M/20$ concentration, but was inhibited to an appreciable degree by $M/10$ salicylate. Cinchophen restricted the O_2-uptake even at $M/50$ concentration. Anaerobic acid production remained unchanged in the presence of $M/10$ salicylate, but was diminished by $M/20$ cinchophen. Acetylsalicylate was rapidly hydrolysed by rat liver and kidney and to a smaller extent also by other animal tissues and fluids as well as by yeast.

The ability of the rat liver to form conjugated glucuronides in vitro was negligible with salicylate and negative with cinchophen, but positive results were obtained with benzoate, *o*- and *p*-aminobenzoate, *p*- and *m*-hydroxybenzoate.

The activity of various animal, plant and yeast enzyme systems was studied. Those affected more by salicylate than by cinchophen were xanthine oxidase, dismutation between hexosediphosphate and pyruvate, carboxylase: cinchophen acted more strongly than salicylate on indophenol oxidase, certain dehydrogenases, glucose fermentation in yeast.

Experiments on the intact animal were done by injecting various doses of salicylate and cinchophen as well as certain benzoic acid derivatives other than salicylate. The behaviour of the injected animals is described. Tissues, blood and urine were examined at 4, 7 and 24 hr. intervals after the injections.

The respiration of liver slices and the dismutation of hexosediphosphate + pyruvate in muscle were not affected by injections of salicylate and cinchophen. There were remarkable changes in liver glycogen. It disappeared almost completely 4–7 hr. after salicylate injections, but was found fully restored, or even above the control value, 24 hr. later. The behaviour of liver glycogen in response to the injection of cinchophen as well as to benzoic acid derivatives other than salicylate, is also described.

Quantitative estimation of reduction in urine showed a considerable increase in the reducing power of urine after the injection of salicylate, several other derivatives of benzoic acid, or cinchophen. There are indications that the reducing substance in salicylate, but not in cinchophen urine, is ascorbic acid. Bicarbonate, albumen and glucose, but not glucuronide, were found in urine after large doses of salicylate; appreciable amounts of glucuronide were present in urine after the injection of benzoate, anthranilate, *p*-aminobenzoate, *m*- and *p*-hydroxybenzoate.

Substances, which on addition of alkalis (NH_3, NaOH, pyridine) and in presence of O_2 give dark-coloured pigments, were found in urine after treatment with salicylate, anthranilate and *o*-cresotinate, but not after cinchophen or any other of the benzoic acid derivatives examined. It is thought unlikely that the alkali-susceptible substance found in urine during salicylate treatment is alkapton, because its excretion was not suppressed by large doses of ascorbic acid, which, however, abolished completely the excretion of homogentisic acid in alkaptonuria.

The estimation of reducing substances in tissues (liver, kidney, intestine) revealed a nearly twofold increase in liver glutathione content 24 hr. after the injection of salicylate.

Salicylic aldehyde did not cause an increase in the reducing power of urine or the excretion of the alkali-susceptible substance until it was transformed by the organism into salicylic acid.

The author owes much gratitude to Mr S. Williamson and Miss R. Leader for their most valuable assistance throughout this work. This investigation constitutes a part of a scheme of research work approved by the Empire Rheumatism Council.

REFERENCES

Abbassy, M. A., Gray-Hill, N. & Harris, L. J. [1936]. *Lancet*, **2**, 1413.
Birch, T. W., Harris, L. J. & Ray, S. N. [1933]. *Biochem. J.* **27**, 590.
Briggs, A. P. [1922]. *J. biol. Chem.* **51**, 453.
Bueding, E. & Wortis, H. [1940]. *J. biol. Chem.* **133**, 585.
Dixon, M. & Lutwak-Mann, C. [1937], *Biochem. J.* **31**, 1347.
Dixon, M. & Thurlow, S. [1924]. *Biochem. J.* **18**, 971.
Dohrn, M. [1912]. *Biochem. Z.* **43**, 240.
Emmerie, A. & Eekelen, M. van [1934]. *Biochem. J.* **28**, 1153.
Friedemann, T. E., Cotonio, M. & Shaffer, P. A. [1927]. *J. biol. Chem.* **73**, 335.
Green, D. E., Herbert, D. & Subrahmanyan, V. [1941]. *J. biol. Chem.* **138**, 127.
Green, D. E., Needham, D. M. & Dewan, J. G. [1937]. *Biochem. J.* **31**, 2327.
Hagedorn, H. C. & Jensen, B. N. [1923]. *Biochem. Z.* **135**, 46.
Hopkins, F. G. & Morgan, E. J. [1936]. *Biochem. J.* **30**, 1446.
Lipschitz, W. L. & Bueding, E. [1939]. *J. biol. Chem.* **129**, 333.
Lutwak-Mann, C. [1940]. *Biochem. J.* **34**, 517.
—— [1941]. *Biochem. J.* **35**, 610.
Mozołowski, W. [1940]. *Biochem. J.* **34**, 823.
Quick, A. [1926]. *J. biol. Chem.* **69**, 549.
Papageorge, E. & Lewis, H. B. [1938]. *J. biol. Chem.* **123**, 211.
Robertson, W. v. B., Ropes, M. W. & Bauer, W. [1941]. *Biochem. J.* **35**, 903.
—— —— —— [1942]. *Proc. Soc. Exp. Biol., N.Y.*, **49**, 697.
Skorczewski, W. & Sohn, J. [1911]. *Wien. klin. Wschr.* **49**, 113.
Theis, R. C. & Benedict, S. R. [1924]. *J. biol. Chem.* **61**, 67.

83. Adenosine- and Inosine-nucleotides in the Phosphorus Metabolism of Muscle

By **Arnošt Kleinzeller**, *Biochemical Laboratory, Cambridge*

(*Received 19 August 1942*)

It is generally assumed that the adenosine-nucleotides play the main part in the intermediary P metabolism in muscle, whilst the inosine-nucleotides are of minor importance [Meyerhof, Lohmann & Meyer, 1931; Meyerhof & Lohmann, 1932; Lohmann, 1931*b*; 1932]. There has even been controversy about the existence of inosine-triphosphate, first prepared by Lohmann [1932]. In this paper the preparation of inosine-triphosphate, and the isolation of inosine-diphosphate, hitherto unknown, are reported. The results of a comparative study of the roles of adenosine- and inosine-nucleotides in some component reactions of muscle metabolism are described and discussed.

Experimental methods

(1) *Preparation of adenosine-triphosphate (ATP).* The ATP was prepared from fresh horse muscle by the modification of Lohmann's method described by D. M. Needham [1942].

(2) *Preparation of inosine-triphosphate (ITP).* The preparation of ITP by deamination of ATP with HNO_2 [Lohmann, 1932] was confirmed by Mozołowski & Sobczuk [1933] and Kiessling [1934], whilst Barenscheen, Braun & Filz [1933] contested the existence of the nucleotide, and more recently Wagner-Jauregg [1936] was unable to obtain a pure preparation. It was found in the present work that the deamination of ATP with HNO_2 proceeds smoothly without splitting off of inorganic P when the reaction is carried out in acetate buffer at *p*H approximately 4.

2 g. Ba-ATP were converted into the Na-salt and the solution was brought to a volume of 32 ml. 4·52 g. $CH_3COONa.3H_2O$, 10 ml. glacial acetic acid, and 20 ml. 60% $NaNO_2$ were added. The *p*H of the solution was 3·96 (glass electrode). After standing 5–6 hr. at room temperature the solution was cooled with ice and 2*N* NaOH was added until the *p*H was 6–6·5; 12 ml. 3*N* $BaCl_2$ were added, and the precipitation of the Ba-salt of ITP was completed by addition of 2 vol. of ethanol. The precipitate was spun off, suspended in 50 ml. H_2O, and 3*N* HCl was added to the ice-cold suspension to bring the final concentration of HCl to 0·1*N* (as described by Kerr & Seraidarian [1941] for the preparation of ATP). The insoluble fraction was centrifuged off, and re-extracted with 20 ml. 0·1*N* HCl. 1 g. urea was added to the combined centrifugates to destroy the remaining HNO_2 and the liquid was kept in the ice-chest for 1–2 hr. The nucleotide was then precipitated by addition of 25 ml. 25% Ba-acetate and an equal volume of ethanol. The precipitate was spun off, redissolved in 0·1*N* HNO_3 as described above, and precipitated with Lohmann's reagent (as used for the preparation of ATP [Lohmann, 1931*a*]). The Hg-compound was decomposed with H_2S, and the nucleotide was precipitated twice as the Ba-salt by addition of excess Ba-acetate and an equal volume of ethanol to the solution of ITP in 0·1*N* HCl. The Ba-ITP was then washed twice with H_2O, twice with 50% ethanol, twice with absolute ethanol, once with ether and dried in vacuo over $CaCl_2$. The yield varied between 50 and 60% of the theoretical amount. The compound contained $7H_2O$ (found H_2O 14·3%; calculated 13·9%) which it lost by drying in vacuo over P_2O_5 at 100°. The anhydrous compound is very hydroscopic. Analysis: found $7_{min.}$ P 7·62%; N (Kjeldahl) 7·07%; ratio N/$7_{min.}$ P 2·05; ratio total P/$7_{min.}$ P 1·50: calculated $7_{min.}$ P 7·98%; N 7·20%; ratio N/$7_{min.}$ P 2·00; ratio total P/$7_{min.}$ P 1·50.

(3) *Preparation of myosin.* The myosin was prepared from rabbit muscle as described by Bailey [1942], but using 0·5 *M* LiCl containing 0·03 *M* $NaHCO_3$ as extractant, and reprecipitating twice from a 0·5 *M* NaCl solution. For further details see Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham & Shen [1942].

(4) *Preparation of dialysed muscle acetone powder.* Muscle extract [Meyerhof & Kiessling, 1935; see also D. M. Needham & Pillai, 1937] after standing 3 hr. at room temperature was dialysed 5 days at 0° against distilled water (to remove the nucleotides) and precipitated with 3 vol. ice-cold acetone. The precipitate was immediately washed with acetone, then with ether, dried, and stored. Before use a 5% suspension of the finely ground powder was prepared, and after a few minutes standing the liquid was centrifuged and the clear supernatant was used.

(5) *Preparation of muscle extract* [Ostern, Guthke & Terszakowec, 1936]. Fresh, minced rabbit muscle was extracted for 10 min. at 0° with 1·5 vol. water and strained through muslin. The extract was cleared by filtering through filter-paper pulp, solid $NaHCO_3$ was added to bring the *p*H to 7 (bromothymol blue), the liquid was dialysed for 6 hr. against running tap water, and left to autolyse at 20° for 90 min. The extract was kept in the ice chest and used within 48 hr.

Results

(1) *Splitting of ATP and ITP by myosin.* It is generally assumed that the first step in the series of chemical changes accompanying muscle contraction is the breakdown of ATP to ADP and PO_4''' [D. M. Needham, 1937; 1942], and recent work [Szent-Györgyi & Banga, 1941; D. M. Needham, 1942; Bailey, 1942; J. Needham, Kleinzeller, Miall, Dainty, Needham & Lawrence, 1942] added evidence in favour of the view [Engelhard & Liubimova, 1939] that the enzyme effecting this split of ATP is myosin itself. Since it was found [J. Needham *et al.* 1942] that inosine-triphosphate affects the flow-birefringence of myosin solutions to the same extent as ATP, the rates of splitting of PO_4''' from ATP and ITP by myosin solutions were compared. Each test-tube contained 0·11–0·18 mg. myosin N (Kjeldahl), 0·1 ml. 18 *N* $CaCl_2$, 0·5 mg. $7_{min.}$ P ATP or ITP (Na-salt), 1 ml. 0·1 *M* buffer solution (glycine or veronal buffer), and the volume was adjusted to 2 ml. with 0·5 *M* NaCl. After varying times of incubation at 37·5° the reaction was stopped by addition of 2 ml. 4% CCl_3COOH and inorganic P was estimated in an aliquot.

As shown in Fig. 1 and Table 1, ITP is split approximately 3 times as fast as ATP by myosin solutions. These results were obtained at *p*H 6·7 and 7·2 (veronal buffer), and at *p*H 9·1 (glycine buffer) with two different myosin preparations. As with ATP, myosin splits off only 50% of the $7_{min.}$ P of the ITP added.

(2) *Preparation of inosine-diphosphate (IDP).* As shown in Fig. 1 and Table 1, in the presence of myosin one PO_4 radical is liberated from each molecule of ITP. The inosine-diphosphate formed was isolated in the following way [see preparation of adenosine-diphosphate from ATP, Liubimova & Pevzner, 1941; Bailey, 1942]: 1 g. Ba-ITP (air-dry) was converted into the Na-salt and the solution was made up to 50 ml. A solution was prepared of myosin in 0·1 *M* glycine buffer *p*H 9·1, containing 62·7 mg. protein and 200 mg. $CaCl_2$ in 150 ml. After bringing both solutions to 37·5° they were mixed and incubated for 10 min. at 37·5°. This time was found to be sufficient to bring about a 50% splitting of the $7_{min.}$ P of the ITP present (cf. Table 1). After incubation the myosin was precipitated by acidifying the solution with 2 *N* HCl to *p*H 5, the mixture was centrifuged, and the clear ice-cold supernatant was brought to *p*H 6 with *N* NaOH. A slight precipitate, 70 mg. dry weight, which formed on addition of 3 ml. 25% Ba-acetate was centrifuged off, and after neutralization of the clear liquid to *p*H 7 the IDP was precipitated by excess of Ba-acetate and 50% ethanol. The precipitate was suspended in H_2O, 3 *N* HNO_3 was added to the final concentration of 0·1 *N*, and the IDP was reprecipitated from the clear

solution by excess of Ba-acetate and 50% ethanol. The precipitate was redissolved in 0·1*N* HNO_3 as described above and precipitated with Lohmann's reagent. The mercury compound was decomposed with H_2S, the clear filtrate freed of H_2S by aeration, and the IDP twice precipitated from 0·1*N* HNO_3 solution with excess of Ba-acetate and 50% ethanol. The Ba-IDP was washed with 50% ethanol until the washings were free of Ba^{++}

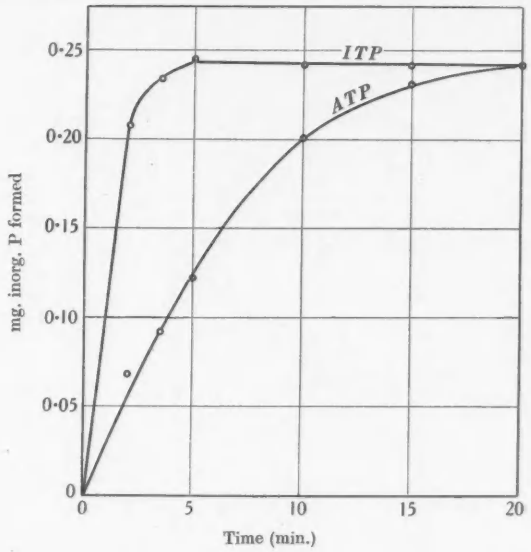


Fig. 1. Splitting of adenosine-triphosphate and inosine-triphosphate by myosin. 0·1*M* veronal buffer *p*H 7·2.

Table 1. *Splitting of ATP and ITP by myosin*

Exp. 1. 0·1*M* veronal buffer *p*H 6·7 in 0·5*M* NaCl. Exp. 2. 0·1*M* glycine buffer *p*H 9·1.

Exp. no.	Myosin N in tube mg.	Initial $7_{min.}$ P mg.	Duration of exp. min.	Inorg. P found mg. ATP	Inorg. P found mg. ITP
1	0·18	0·500	5	0·053	0·194
			10	0·128	0·205
			15	0·142	0·208
			20	0·173	0·215
2	0·108	0·509	2	0·080	0·200
			3½	0·117	0·237
			5	0·156	0·255
			10	0·217	0·250
			15	0·246	0·259
			20	0·248	0·250

(tested with Na_2SO_4), with 75% ethanol, twice with 96% ethanol, once with ether, and dried in vacuo over $CaCl_2$. Yield 0·625 g., i.e. 68% of theory. Analysis: found 14·4% H_2O; 4·08% $7_{min.}$ P; 8·25% total P; 7·29% N (Kjeldahl): calculated for $C_{10}H_{11}O_{11}N_4P_2Ba_{1·5}\cdot 6H_2O$, 14·6% H_2O; 4·20% $7_{min.}$ P; 8·39% total P; 7·05% N. Ba in the anhydrous compound (Weiler): found 31·6%; calculated for $C_{10}H_{11}O_{11}N_4P_2Ba_{1·5}$, 32·6%.

(3) *Phosphagen formation in the presence of adenosine- and inosine-nucleotides.* Meyerhof & Lohmann [1932] have shown that the formation of phosphagen in muscle extracts proceeds also in the presence of ITP. In our experiments the rates of P transfer from

phosphoglycerate to creatine (Parnas reaction) in the presence of ATP, ITP, adenosine-diphosphate (ADP), and inosine-diphosphate (IDP) were compared. Acetone powder prepared from dialysed muscle extract (see above) was used as the enzymic system. Each tube contained 0·2 ml. 5% enzyme solution, 0·2 ml. 2% $NaHCO_3$, 6 mg. creatine, 0·6 mg. Mg^{++} (as $MgCl_2$), 1·2 mg. P of Na-phosphoglycerate, and 0·29 mg. $7_{min.}$ P ATP or ITP (or equimolecular quantities of the corresponding diphosphates). The volumes of the

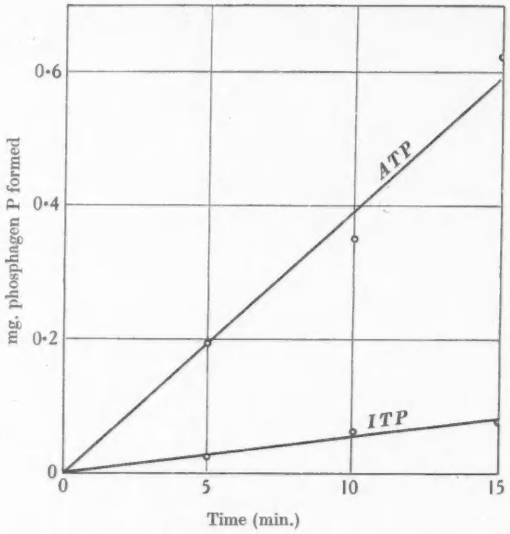


Fig. 2. The formation of phosphagen in the presence of ATP and ITP.

Table 2. *The formation of phosphagen in the presence of adenosine- and inosine-nucleotides*

Nucleotide	mg. $7_{min.}$ P added	Duration of exp. min.	Phosphagen P found mg.	Nucleotide	mg. $7_{min.}$ P added	Duration of exp. min.	Phosphagen P found mg.
ADP	0·181	5	0·192	IDP	0·181	5	0·009
		10	0·242			10	0·032
		15	0·318			15	0·048
ATP	0·291	15	0·287	ITP	0·292	15	0·058

contents of the test tubes were adjusted to 2 ml. with H_2O. After varying times of incubation at 40° the test tubes were transferred to a freezing mixture in order to stop the reaction, and the contents were deproteinized by addition of 2 ml. 8% trichloroacetic acid. The phosphagen content was estimated as described by Baldwin & D. M. Needham [1937]. In control experiments no phosphagen formation was found in the absence of ATP.

As shown in Fig. 2 and Table 2 the formation of phosphagen is 5–8 times as fast in the presence of ATP as in the presence of ITP. No appreciable difference was found in the rate of formation of phosphagen between the respective triphospho- and diphospho-nucleotides (see Table 2).

(4) *Phosphorylation of hexosemonophosphate.* It was shown by Ostern, Guthke & Terszakowec [1936], and Sakov [1941] that muscle extract contains an enzyme which transfers P from ATP to hexosemonophosphate (fructose-6-phosphate) with formation

of fructose-diphosphate. In our experiments the phosphorylation of hexosemonophosphate in the presence of ATP and ITP was examined; Robison ester (glucose-6-phosphate) was used, since the muscle extract contains an enzyme converting this into an equilibrium mixture of fructose- and glucose-6-phosphate. Iodoacetic acid was added to stop the reaction at the fructose-diphosphate and triose-phosphate stage. 5 ml. muscle extract, 4·5 ml. H_2O, 1 ml. Robison ester (6 mg. P), 0·3 ml. 0·5 *M* $MgSO_4$, 0·2 ml. 0·1 *M* iodoacetate, 1·4 ml. 0·1 *M* veronal buffer *p*H 7·0, and 2 ml. ATP or ITP (2·50 mg. $7_{min.}$ P) were incubated at 20°. At varying intervals 2 ml. aliquots were pipetted into 0·6 ml. 10% CCl_3COOH and in aliquots of the centrifugate inorganic P and $7_{min.}$ P were estimated. As shown in Table 3, there is a rapid disappearance of $7_{min.}$ P in the presence of ATP, whilst only dephosphorylation of ITP is observed. No evidence was therefore obtained that ITP can transfer P to hexosemonophosphate. (It should be noted that the disappearance of $7_{min.}$ P in these conditions gives only a rough estimate of the ATP disappearing, since the hexose-diphosphate and triose-phosphate are to some extent split.) The rapid formation of NH_4^+ from ATP in presence of hexosemonophosphate, observed by Ostern *et al.* [1936], is therefore due to a deamination of the adenylic acid formed during the reaction.

Table 3. *Phosphorylation of hexosemonophosphate in the presence of ATP and ITP*

2 ml. aliquots.

Time min.	ATP		ITP	
	Inorg. P found mg.	$7_{min.}$ P found mg.	Inorg. P found mg.	$7_{min.}$ P found mg.
0	0·067	0·372	0·087	0·368
5	0·090	0·301	0·107	0·374
10	0·102	0·240	0·130	0·365
20	0·102	0·247	0·159	0·365

The results in Table 3 show that a rapid dephosphorylation of ATP and ITP takes place in the muscle extract. This is due to an enzyme which [Sakov, 1941] seems not to be identical with myosin. It was therefore thought of interest to examine the dephosphorylation of adenosine- and inosine-nucleotides in muscle extract. 5 ml. muscle extract, 0·1 ml. 0·1 *M* iodoacetate (to prevent rephosphorylation of the nucleotides by means of inorganic P), and 0·6 ml. 0·1 *M* veronal buffer *p*H 7·0 were incubated at 20° with 1 ml. of solutions of the respective nucleotides. 1 ml. aliquots were pipetted at varying intervals into 1 ml. 5% CCl_3COOH and in the deproteinized centrifugates inorganic P was estimated. As shown in Table 4, the formation of inorganic P increases linearly with time in the presence of ATP and ADP. ITP is split only partially, whilst IDP is not dephosphorylated at all.

Table 4. *Dephosphorylation of adenosine- and inosine-nucleotides in muscle extract*

1 ml aliquots.

Exp. no.	Time min.	mg. $7_{min.}$ P added	Inorganic P formed	
			ATP	ITP
1	5	0·170	0·018	0·016
	10		0·050	0·037
	15		0·073	0·048
	20		0·099	0·053
			ADP	IDP
2	5	0·106	0·018	0
	10		0·044	0
	15		0·065	0
	20		0·096	0

From these experiments it can be concluded that the dephosphorylation of ITP stops at the IDP stage. This suggests that the myokinase [Kalckar, 1942] which catalyses the reaction:

$$2\ \text{ADP} \rightarrow \text{adenylic acid} + \text{ATP}$$

is strictly specific for the adenosine compounds.

(5) *Phosphorylation of glucose by yeast hexokinase.* The rate of phosphorylation of glucose by yeast hexokinase in the presence of ATP and ITP was measured. The enzyme solution was prepared by grinding 30 mg. of the dry powdered hexokinase with 4 ml. H_2O; the suspension was left standing for a few minutes and spun off. The clear supernatant was used.

Test-tubes containing 0·8 ml. enzyme solution, 2·2 mg. $7_{min.}$ P of ATP or ITP, 2 ml. 0·4 *M* glucose, 2 ml. 0·1 *M* veronal buffer *p*H 7·8, 0·4 ml. 0·5 *M* $MgSO_4$, and H_2O to bring the volume to 8 ml., were incubated at 37°. At varying intervals 1 ml. aliquots were

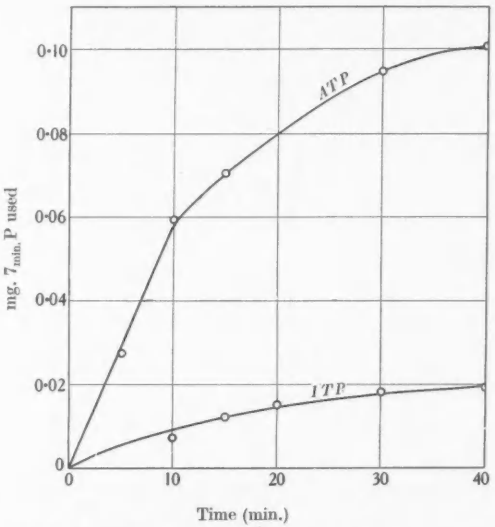


Fig. 3. Phosphorylation of glucose in the presence of ATP and ITP.

pipetted into 1 ml. 2 *N* HCl. The $7_{min.}$ P was now estimated in the aliquots. The difference between the initial $7_{min.}$ P value and that after incubation indicates the amount of glucose phosphorylated.

As shown in Fig. 3, glucose is phosphorylated in the presence of ATP approximately 5 times as fast as in the presence of ITP.

Discussion

The experimental results show that the ITP and IDP are considerably less effective than the respective adenosine-nucleotides in the P transfer reaction of Parnas, and in the phosphorylation of glucose by yeast hexokinase; no evidence of the transfer of P from ITP to hexosemonophosphate was obtained; on the other hand, myosin splits PO_4''' more readily from ITP than from ATP. Inosine-triphosphate has been isolated from frog muscle [Lohmann, 1932], hence it may be assumed that ITP can be formed during

muscle contraction. The formation of NH_3 during muscle contraction seems to be connected with the liberation of PO_4''', although the views of different schools do not so far conform as to whether the formation of NH_4^+ precedes, is parallel to, or follows that of PO_4''' [see Mozołowski, Reiss & Sobczuk, 1932]. The mother substance of both NH_3 and H_3PO_4 is ATP. The higher rate of splitting of PO_4''' from ITP than from ATP by myosin solutions may thus have a bearing on the present conception of P metabolism in muscle during the first phase of contraction.

It is known that during aerobic muscle contractions the content of adenosine-nucleotides present in the muscle does not much decrease [Parnas, 1929]. If it is assumed that the inosine-nucleotides play a greater part in the P metabolism of muscle than was thought hitherto, their reamination would be expected. This reamination was postulated by Embden, Riebeling & Selter [1928] and Parnas [1929], but the suggestions advanced for its mechanism are controversial. Our attempts to find evidence of reamination of ITP or IDP to the corresponding adenosine compounds in muscle extracts have as yet been unsuccessful. Experiments were set up incubating ITP or IDP with aminoacids or aminoacid-amides in the presence of dialysed muscle extract (rabbit muscle extract as described by Meyerhof & Kiessling [1935] but not precipitated with acetone). This extract readily deaminated ATP, and the deamination was not inhibited by the presence of the added aminoacids. If reamination of the inosine-compounds had occurred, it would have been indicated by formation of NH_4^+. In our experiments no NH_4^+ was formed when ITP or IDP was incubated together with alanine, glutamate, glutamine, aspartate, asparagine, or ornithine.

Summary

1. The preparation of inosine-triphosphate (ITP) is described.

2. The rates at which PO_4''' is liberated from inosine-triphosphate and adenosine-triphosphate by myosin solutions are compared. Inosine-triphosphate is split approximately 3 times as fast as ATP.

3. The isolation of the hitherto unknown inosine-diphosphate (IDP) is described.

4. Phosphagen formation proceeds 5–8 times as fast in the presence of adenosine-nucleotides as in the presence of the inosine-compounds.

5. No evidence of transfer of P from ITP to hexosemonophosphate was obtained.

6. It is shown that the dephosphorylation of ITP in muscle extracts stops at the IDP stage. It is concluded that myokinase is strictly specific for adenosine-diphosphate.

7. The phosphorylation of glucose by yeast hexokinase proceeds approximately 5 times as fast in the presence of adenosine-triphosphate as in the presence of ITP.

The author wishes to express his thanks to Dr D. M. Needham and Dr J. Needham, F.R.S., for their advice and criticism, and also to Prof. Sir F. G. Hopkins, O.M., and Prof. E. Friedmann for their interest and encouragement. The author gratefully acknowledges a grant from the Rockefeller Foundation which made the above work possible.

REFERENCES

Bailey, K. [1942]. *Biochem. J.* **36**, 121.
Baldwin, E. & Needham, D. M. [1937]. *Proc. roy. Soc.* B, **122**, 197.
Barenscheen, H. K., Braun, K. & Filz, W. [1933]. *Biochem. Z.* **265**, 141.
Dainty, M., Kleinzeller, A., Lawrence, A. S. C., Miall, M., Needham, J., Needham, D. M. & Shen, S. C. [1942]. *Proc. roy. Soc.* B (in the Press).
Embden, G., Riebeling, C., & Selter, G. E. [1928]. *Hoppe-Seyl. Z.* **179**, 149.
Engelhard, W. A. & Liubimova, M. N. [1939]. *Nature, Lond.*, **144**, 668.
Kalckar, H. [1942]. *J. biol. Chem.* **143**, 299.
Kerr, S. E. & Seraidarian, K. [1941]. *J. biol. Chem.* **139**, 121.
Kiessling, W. [1934]. *Biochem. Z.* **273**, 103.
Liubimova, M. N. & Pevzner, D. [1941]. *Biochemica*, **6**, 178.

Lohmann, K. [1931*a*]. *Biochem. Z.* **233**, 460.
—— [1931*b*]. *Biochem. Z.* **237**, 445.
—— [1932]. *Biochem. Z.* **254**, 381.
Meyerhof, O. & Kiessling, W. [1935]. *Biochem. Z.* **283**, 83.
Meyerhof, O. & Lohmann, K. [1932]. *Biochem. Z.* **253**, 421.
Meyerhof, O., Lohmann, K. & Meyer, K. [1931]. *Biochem. Z.* **237**, 437.
Mozołowski, Wł., Reiss, J., & Sobczuk, B. [1932]. *Biochem. Z.* **249**, 157.
Mozołowski, Wł. & Sobczuk, B. [1933]. *Biochem. Z.* **265**, 41.
Needham, D. M. [1937]. Article in Perspectives in Biochemistry. Camb. Univ. Press.
Needham, D. M. [1942]. *Biochem. J.* **36**, 113.
Needham, D. M. & Pillai, R. K. [1937]. *Biochem. J.* **31**, 1837.
Needham, J., Kleinzeller, A., Miall, M., Dainty, M., Needham, D. M. & Lawrence, A. S. C. [1942]. *Nature, Lond.*, **150**, 46.
Ostern, P., Guthke, J. A. & Terszakowec, W. [1936]. *Hoppe-Seyl. Z.* **243**, 9.
Parnas, J. K. [1929]. *Biochem. Z.* **206**, 16.
Sakov, N. E. [1941]. *Biochemica*, **6**, 163.
Szent-Györgyi, A. & Banga, I. [1941]. *Science*, **93**, 158.
Wagner-Jauregg, T. [1936]. *Hoppe-Seyl. Z.* **239**, 188.

84. Intercellular Hormones

5. Evidence that the Proliferation Promoting Effect of Damaged-Cell Products is Attributable to Adenine Nucleotides and known Growth Factors

By **John R. Loofbourow**, *Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.*

(*Received 5 August 1942*)

Evidence was presented [Loofbourow, 1942*a*] that the mechanism whereby proliferation-promoting (PP) factors appear in the intercellular fluids when cells are slightly damaged involved three steps: (1) an increase in cell-membrane permeability, (2) a diffusion of non-protein substances through the more permeable membranes into the intercellular fluids, and (3) a synthesis within the living, injured cells of substances whose concentration therein is disturbed by diffusion. Such a mechanism might be expected to result in the presence in the intercellular fluids of a variety of non-protein growth-stimulating materials rather than of a single active substance. The investigations reported here indicate that the release of PP factors by damaged cells does involve a multiplicity of active substances, among which are adenine nucleotides and certain known growth factors.

Experimental

The preparation of intercellular fluids has been previously described in detail [Loofbourow, Webb, Loofbourow & Abramowitz, 1942]. As in the earlier experiments, the organism employed was yeast (*S. Cerevisiae*, F.B. strain) suspended at 100 g./l. in distilled H_2O, the injuring agent was lethal ultraviolet radiation, and the suspensions were irradiated for 8 hr. under such conditions that the cells were exposed repeatedly to sublethal doses of radiant energy and were damaged slowly. The PP activity of the preparations was assayed on yeast cultured in roller tubes [Loofbourow *et al.* 1942]. Characteristic properties of a typical preparation are listed in Table 1.

Table 1. *Characteristics of intercellular fluids from damaged and normal yeast cells*

(Cell-free suspension fluids from 100 g. (wet weight) yeast per l. distilled H_2O.)

	I. Suspension fluid from damaged cells	II. Suspension fluid from normal cells
Colour	Clear light straw	Water clear
Fluorescence excited by 3650 Å. radiation:		
Colour	Yellow-green	Blue
Intensity*	42·8	9·5
Ultraviolet absorption spectrum†	Marked maximum at 2600 Å., $D=7{\cdot}0$. No maxima characteristic of proteins, tyrosine, or tryptophane	Maximum at 2600 Å., $D=1{\cdot}4$. No maxima characteristic of proteins, tyrosine, or tryptophane
Solid content, mg./ml.	3·17	0·245
Biuret and phosphotungstic acid tests for protein	Negative	Negative
Potency,‡ growth units per ml.	98·0	3·3
Growth units per mg.	30·9	13·5

* Referred to quinine sulphate, 0·27 mg./l. in 0·1 *N* H_2SO_4, as 48·0 (all readings net, after subtraction of blanks).

† $D=1/x \log_{10} I_0/I_x$.

‡ One growth unit represents an increased 24 hr. yeast crop of approximately 1000% in comparison with the controls [Loofbourow, Dwyer & Cronin, 1941].

Presence in the preparations of heat-labile as well as heat-stable PP factors. Autoclaving for 20 min. at 15 lb. at *p*H 5·4–6·0 caused a loss in activity with regard to the later periods (30–70 hr.) of the growth tests of about 10 % in damaged-cell preparations and about 25 % in normal-cell extracts (Table 2). This effect was overlooked in earlier assays

Table 2. *Effect of autoclaving for 20 min. at 15 lb. on PP activity of intercellular fluids from injured and normal cells*

Material tested for proliferation promoting effect (at 0·1 ml./ml. of culture)	Yeast crop, mg. (wet weight) per ml. after hr.					
	0	16	24	30	45	60
I. Injured-cell extracts:						
Unautoclaved	0·064	1·55	4·05	4·95	5·53	5·55
Autoclaved	0·064	1·55	4·05	4·76	4·95	4·95
II. Normal-cell extracts:						
Unautoclaved	0·064	0·47	0·84	1·00	1·30	1·39
Autoclaved	0·064	0·42	0·70	0·82	0·97	1·06
Controls	0·064	0·07	0·12	0·12	0·12	0·12

[Loofbourow, Dwyer & Morgan, 1938] because they were based entirely on 24 hr. yeast crops. No further loss in activity could be detected on repeatedly re-autoclaving; the tests for further loss in activity involved as many as nine successive assays extending over periods up to 3 months. Exposure of the autoclaved preparations to diffuse daylight during storage caused no measurable change in activity. After storage for approximately 3 months, an autoclaved damaged-cell preparation was re-autoclaved for 65 min. at 15 lb. in 0·05 *M* H_3PO_4, 0·05 *M* NaOH, and at *p*H 5·5, 6·8, and 9·6 without further loss in activity as exhibited in growth periods up to 72 hr.

These results indicated that the preparations contained heat-labile factors responsible in part for the PP effect of non-autoclaved preparations in the growth period after 24 hr., as well as heat-stable factors responsible for the early PP effect and for most of the later growth response.

Presence in the preparations of heat-stable factors other than nucleotides. Close correlation was found between the ultraviolet absorption at 2600 Å., characteristic of nucleotides, and the PP potency of intercellular fluids from damaged rat-embryo tissues [Loofbourow, Dwyer & Lane, 1940] and from damaged yeast cells suspended in isotonic saline [Cook, Loofbourow & Stimson, 1939]. In more recent experiments, suspension fluids from damaged yeast cells in distilled H_2O did not show such correlation (Fig. 1). It appeared, therefore, that the PP activity of damaged-yeast preparations could not be accounted for by the ultraviolet absorbing moiety alone. Against the possibility that the absorbing moiety played no part in the PP effect was the fact that crude preparations, and fractions thereof, having $E^{1\%}_{1\,cm.} = 10$ to 15 at λ 2600 Å., generally had almost inappreciable activity, especially in the early growth periods. A more likely interpretation seemed to be that the crude preparations contained a multiplicity of active factors

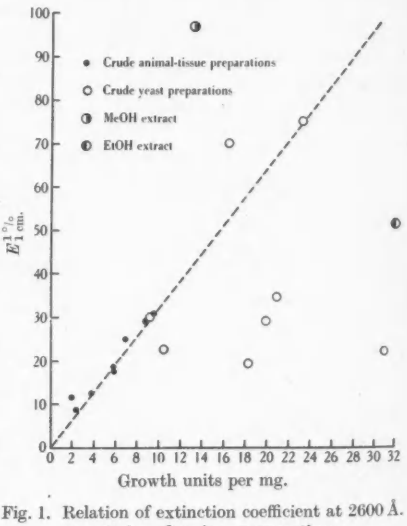

Crude animal-tissue preparations
Crude yeast preparations
MeOH extract
EtOH extract
$E^{1\%}_{1\,cm.}$
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Growth units per mg.

Fig. 1. Relation of extinction coefficient at 2600 Å. to activity of various preparations.

among which the absorbing moiety ceased to be limiting for yeast growth when it reached a level corresponding approximately to $E^{1\%}_{1\,cm.} = 20$ at λ 2600 Å.

Various fractionation procedures failed to result in proportionate concentration of PP factors and ultraviolet absorbing substances in the same fractions. In early experiments [Stimson, Aquinas & Loofbourow, 1940], crude damaged-yeast preparations, both before and after reduction in volume by vacuum distillation and precipitation of inactive material with hot 95 % ethyl alcohol, were passed through adsorption columns made up of equal parts of magnesium oxide and Filtercell. In 3650 Å. radiation, a brilliant fluorescing band regularly appeared at the top of the column. This could be developed into three bands, the top one fluorescing yellow, the middle one white, and the lower one blue. The filtrates fluoresced blue. Various portions of the columns were eluted with boiling H_2O, and the PP activity and ultraviolet absorption of the eluted fractions and filtrates were determined. It was thought at first that the major portion of the PP activity and ultraviolet absorbing moiety accumulated together in the yellow-fluorescing band. Further experiments indicated, however, that the activity, but not the ultraviolet absorption of fluorescence, was rather uniformly distributed among the fractions. Interpretation of these results is difficult because of the possibility that the presence of oxidation products may have complicated the observations. Thus, it was noted that when white or blue fluorescing eluates were dried on a water bath, the portion which dried most rapidly often exhibited a change in fluorescent colour to yellow, corresponding to the top bands of the columns. Similar changes from blue to yellow fluorescence were noted in solutions of barbituric acid dried rapidly and redissolved [Joyce, 1940], and a somewhat analogous development of new fluorescent properties occurs when thiamine is oxidized to thiochrome.

In recent experiments, damaged-cell products prepared from yeast in distilled H_2O were fractionated by various solvents. A preparation taken to dryness on a water bath was successively extracted, in a Soxhlet apparatus, with hot ethyl ether, methyl alcohol, and 95 % ethyl alcohol, with the results shown in Table 3. Ether removed approximately 7 % of the material but only a negligible proportion of the PP activity or ultraviolet absorption. The PP factors and ultraviolet absorbing materials were readily soluble in hot methyl or ethyl alcohol, the final residue of insoluble material, which represented about 40 % of the original material, retaining but a small proportion of the activity or absorption. The extinction coefficients of these fractions showed even less correlation with their PP activities than that found for crude preparations (Table 3 and Fig. 1).

Table 3. *Fractionation of injured yeast preparations by successive extractions with hot solvents*

	Fraction by weight, W	$E^{1\%}_{1\,cm.}$ at λ 2650 Å.	$E \times W$	Growth units per mg., G.U.	G.U. $\times W$
I. Crude material	1·00	29·0	29·0	20·0	20·0
II. Ether extract of I	0·072	0·021	0·00	—	—
III. MeOH extract of I	0·514	51·2	26·3	32·0	16·5
IV. EtOH extract of I	0·016	97·0	1·6	13·3	0·2
V. Residue from IV	0·400	3·0	1·2	8·8	3·5
Totals, II–V	1·00	—	29·1 (+0·3 %)	—	20·2 (+1·0 %)

An aliquot of the methyl alcohol extract was dried and extracted successively with hot acetone and 95 % ethyl alcohol. The material was insoluble in acetone. The ultraviolet absorbing moiety divided approximately equally between the ethyl alcohol extract and the residue. The sums of the activities of the fractions did not equal the activity of the original material, however, so that comparison of the extinction coefficients and activities of the fractions was not possible. The apparent loss in activity in the fractions may have

been due to separation of factors which show maximum effectiveness only in combination, or to chemical breakdown. The solubility behaviours of the PP factors was similar to that of bios preparations [Bishop & Rainbow, 1939] and of various known water-soluble growth factors. The solubility behaviour of the ultraviolet absorbing moiety was suggestive of various nucleotides, rather than of nucleic acid or its constituent purines and pyrimidines [Levene & Bass, 1931]. The observed lack of correlation of extinction coefficients and PP activity corresponded to similar results obtained by Cook, Hart & Stimson [1940] with bios fractions from yeast.

From these experiments, it appears that the ultraviolet absorbing moiety of damaged yeast cell products cannot account for the entire PP effect of such preparations.

Presence in the preparations of heat-stable PP factors other than certain known growth factors. To determine whether the PP activity could be attributed entirely to certain known growth factors, two supplements, A and B, were made up in Reader's medium [Reader, 1927]. Supplement A contained the following substances (concentrations in μg. per ml.): thiamine hydrochloride, 2·5; riboflavin, 5·0; pyridoxine, 5·0; calcium pantothenate, 1·25; biotin (free base), 0·00125; choline, 7·5; and nicotinamide, 25·0. Supplement B contained the following amino-acids, each at a concentration of 6·25 mg./ml.: glutamic acid, asparagine, aspartic acid, leucine, and arginine. These concentrations were selected on the basis of data of Williams, Eakin & Snell [1940] to provide an excess of each of the factors when the supplements were added to Reader's medium in the proportion of 1 ml. of supplement in 10 ml. of supplemented medium. The PP effects of intercellular fluids from damaged and undamaged yeast cells were then tested in Reader's medium with and without these supplements.

Cook [1942] had found previously that the addition of preparations from damaged yeast cells gave increased growth in either Reader's or Williams' medium supplemented with thiamine, pyridoxine, pantothenic acid, biotin, inositol, and twenty amino-acids. Preliminary data substantially confirming Cook's results have been reported elsewhere [Loofbourow, 1942*b*]. These data have been extended by further studies with regard to (*a*) concentration effects, and (*b*) growth kinetics. At concentrations of 0·1 ml. each per ml. of culture for supplements A and B and of 0·05 ml./ml. of culture for preparations from damaged and undamaged yeast cells, the increased growth resulting from the addition of damaged-cell products to the supplemented media was clearly apparent (Table 4). When, however, the concentration level of the supplements was increased, the

Table 4. *Effect of adding suspension fluids from damaged and undamaged cells to cultures containing various growth factors and amino-acids*

Added material per ml. of final culture	Yeast crop, mg. (wet weight) per ml. (initial seeding, 0·064 mg./ml.) after hr.					
	9	20	24	33	50	75
None (controls)	0·115	0·147	0·152	0·160	0·166	0·160
0·1 ml. supplement A	0·349	2·57	3·08	3·48	3·85	3·73
0·1 ml. each supplements A and B	0·348	3·57	4·43	5·29	6·07	6·02
0·05 ml. damaged-cell preparation	0·434	3·32	3·70	4·38	4·57	4·43
0·05 ml. damaged-cell preparation plus 0·1 ml. supplement A	0·452	3·57	4·03	4·53	4·90	4·80
0·05 ml. damaged-cell preparation plus 0·1 ml. each supplements A and B	0·367	4·35	5·40	6·77	6·77	6·30
0·05 ml. undamaged-cell preparation	0·309	0·771	0·803	0·891	0·983	1·03
0·05 ml. undamaged-cell preparation plus 0·1 ml. supplement A	0·359	2·63	3·00	3·70	4·07	—
0·05 ml. undamaged-cell preparation plus 0·1 ml. each supplements A and B	0·359	3·44	4·31	5·19	5·89	5·66

effect of adding damaged-cell preparations became less apparent, until at a level of 0·2 ml./ml. of culture for each of the two supplements, almost negligible increased growth resulted from the addition of damaged-cell preparations. This is illustrated in Fig. 2, which, although plotted for 24 hr. growth, is substantially representative of the growth-concentration relationships observed throughout the growth periods from 20 to 70 hr.

The suppression of the PP effect of damaged-cell preparations by high concentrations of the two supplements suggested the presence in the damaged-cell products of at least some of the factors in the supplements. That the damaged-cell products also contained other PP factors was indicated by two results of these experiments. First, greater growth

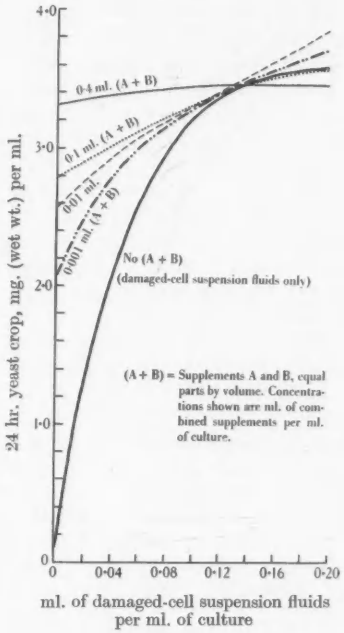

4·0
3·0
2·0
1·0
0
24 hr. yeast crop, mg. (wet wt.) per ml.
0·4 ml. (A + B)
0·1 ml. (A + B)
0·01 ml. (A + B)
0·001 ml. (A + B)
No (A + B)
(damaged-cell suspension fluids only)
(A + B) = Supplements A and B, equal parts by volume. Concentrations shown are ml. of combined supplements per ml. of culture.
0
0·04
0·08
0·12
0·16
0·20
ml. of damaged-cell suspension fluids per ml. of culture

Fig. 2.

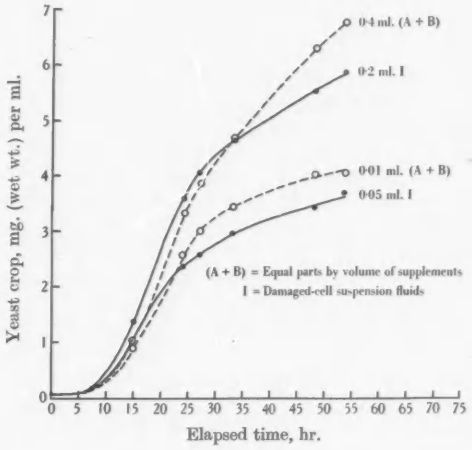

Yeast crop, mg. (wet wt.) per ml.
0·4 ml. (A + B)
0·2 ml. I
0·01 ml. (A + B)
0·05 ml. I
(A + B) = Equal parts by volume of supplements
I = Damaged-cell suspension fluids
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Elapsed time, hr.

Fig. 3.

Fig. 2. Effect on 24-hour yeast crop of adding damaged-cell products to media containing various concentrations of supplements A and B. (Supplement A contained known growth factors and supplement B contained amino-acids. See text for details.)

Fig. 3. Comparison of growth kinetics obtained with damaged-cell products and with supplements A and B.

was obtained with the higher concentrations of damaged-cell preparations and 0·005 ml. of each supplement per ml. of culture than with even the highest concentration of the combined supplements alone. Secondly, the growth kinetics with the combined supplements alone differed from those with the damaged-cell products alone, the latter being characterized by greater initial PP stimulation and less final crop than the former at comparable concentration levels (Fig. 3). These data indicated a qualitative as well as a quantitative difference between the damaged-cell preparations and the supplements. The possibility has to be considered that the qualitative difference might be attributable to variation in relative proportions of the same factors, present in both the supplements and the damaged-cell products, as well as to the presence of additional factors in the damaged-cell products.

Fig. 4 shows 24 hr. growth *v.* concentration-by-weight relationships for the combined supplements and for damaged-cell preparations. Again, a qualitative difference is evident. It should be noted that at the higher concentration levels the crude damaged-cell preparations were more potent, weight for weight, than the combined supplements. This was true despite the fact that the damaged-cell suspension fluids diluted the Reader's medium in proportion to the amount of fluid added, whereas the supplements did not, since the latter were made up in Reader's medium.

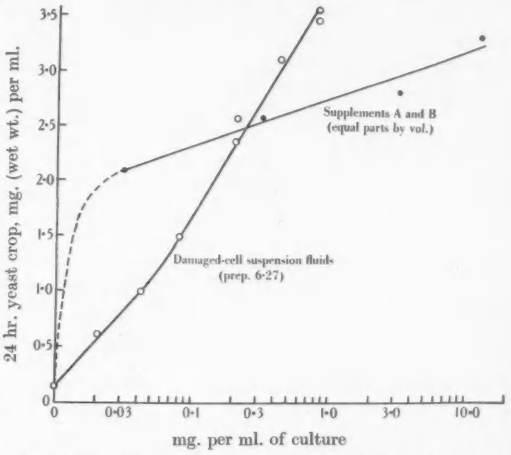


Fig. 4. Comparison of the PP potencies of damaged-cell products and of the combined supplements A and B at various concentration levels.

Concurrently with these investigations, Mr Webb of our laboratory undertook the chemical and microbiological assay of various preparations from damaged and undamaged cells for the presence of certain known growth factors. His results will be published in detail as a subsequent paper in this series; it is necessary to refer to them here in connexion with the following experiments. According to his assays, a typical damaged-yeast preparation contained (in μg./ml.) biotin 0·0125, pyridoxine 1·28, pantothenic acid 2·86, thiamin 0·01, nicotinic acid 5·40, and folic acid 0·0136. A water solution of these factors, in corresponding concentrations, was made up and designated 'supplement C'. A comparison was then made in Reader's medium of the PP effect of C, of a typical damaged-cell preparation (I), and of C and (I) in equal proportions by volume. Supplement C failed to reproduce the PP effect of (I), hence factors other than those in C apparently accounted in part for the PP activity of (I). The mixture of C and (I) exhibited, however, approximately the same PP activity as that of (I), indicating that the additional factors in (I), other than those in C, were present in excess of limiting quantities.

Simulation of the PP effect of damaged-cell products by combinations of known growth factors and adenine nucleotides. Since neither the ultraviolet absorbing moiety alone nor certain known growth factors appeared to account entirely for the PP activity of damaged-cell products, an investigation was undertaken of the combined PP effects of nucleotides and growth factors. Adenine complexes were employed because, as previously reported [Loofbourow *et al.* 1942; Loofbourow, 1942*b*], spectroscopic and chemical evidence indicated that the nucleotide moiety contained adenine. Because of the marked effect of the damaged-cell preparations in stimulating cell respiration, and because of the markedly greater PP effect of damaged-cell preparations when the cultures were well aerated, it had been thought that the nucleotide moiety might be diphospho- or triphosphopyridine nucleotide [Loofbourow *et al.* 1942]. Preliminary evidence (biological tests and effects of hyposulphite reduction on the spectra) indicated that this was not the case, but it is felt that the possibility that a part of the nucleotide absorption is attributable to these coenzymes has not been excluded entirely.

The effects of adenosine, yeast adenylic acid, muscle adenylic acid, and adenosine triphosphate on the growth of yeast in Reader's medium were investigated. The concentrations employed ranged from 5×10^{-6} to $2{\cdot}5 \times 10^{-4}\,M$, after dilution by the Reader's

medium. The extinction coefficients at 2600 Å. of typical damaged-cell preparations were equivalent to adenine molarities of about 5×10^{-4} in the crude preparations. Hence, on this basis, the concentrations of the nucleotides employed corresponded to the equivalent of 0·01–0·5 ml. of damaged-cell preparations per ml. of culture.

None of the nucleotides alone duplicated the activity of damaged-cell preparations in Reader's medium. Yeast adenylic acid appeared to have negligible effect. Adenosine triphosphate, muscle adenylic acid, and adenosine all stimulated proliferation, the effect decreasing in the order listed and being in all instances considerably lower in magnitude that the PP effect of equivalent concentrations of damaged-cell preparations. In the period less than 24 hr., the growth kinetics obtained with the damaged-cell preparations appeared to be most closely simulated by adenosine triphosphate.

Adenosine triphosphate, muscle adenylic acid, and adenosine were then tested in Reader's medium in combination with supplements A and B. Each ml. of culture contained either 0·1 ml. of A or 0·1 ml. each of A and B. At a concentration in the cultures of $5 \times 10^{-5} M$ the three adenyl compounds each caused increased growth response when added to media supplemented with A alone or supplemented with both A and B. At $2{\cdot}5 \times 10^{-4} M$ adenosine triphosphate added to media supplemented with A resulted in essentially the same growth as did a damaged-cell preparation alone at 0·05 ml./ml. of culture (Fig. 5). Furthermore, adenosine triphosphate at this concentration caused essentially the same increase in growth above that obtained with the combined supplements A and B as did the addition to the supplemented media of 0·05 ml. of damaged-cell products per ml. of culture. It was possible, therefore, (*a*) to duplicate the effect of damaged-cell products alone by a combination of known growth factors and adenosine triphosphate, and (*b*) to duplicate the effect of adding damaged-cell products to well-supplemented media by the addition of this nucleotide to similarly supplemented media. It should be pointed out that the concentration of the nucleotide necessary to produce these effects corresponded to 10 times the nucleotide equivalent of the damaged-cell products as computed from their ultraviolet absorption at 2600 Å.

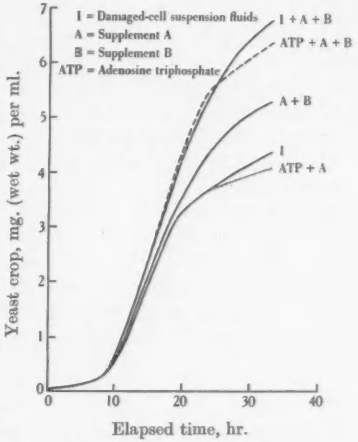


Fig. 5. Duplication of the proliferation-promoting effect of damaged-cell products by adenosine triphosphate and known growth factors.

Discussion

The interrelationships of cells through the intercellular *milieu* appears to be more complex than has previously been realized. When cells are damaged by such widely different agents as radiant energy, mechanical injury, and chemical injury [Loofbourow & Dwyer, 1938; 1939 *a*, *b*; Loofbourow, Cook, Dwyer & Hart, 1939], proliferation-promoting factors and substances capable of markedly stimulating cell respiration [Fardon, Carrol & Ruddy, 1938; Sizer & Loofbourow, 1942] are released into the intercellular fluids. This general type of phenomenon is observed either with animal-tissue cells [Menkin, 1941; Loofbourow, Cook *et al.* 1939; Loofbourow, Cueto & Lane, 1939; Loofbourow *et al.* 1940] or yeast. The phenomenon occurs not only when cells are sufficiently damaged to cause their breakdown and disintegration, but when the cellular injury is insufficient to cause cytolysis. In the latter case, the release of active factors by the living, injured cells appears to be due to increased membrane permeability and to be accompanied by

resynthesis of some of the materials lost from the cells [Loofbourow, 1942*a*]. Subjecting cells to anaerobiosis leads to much the same type of phenomenon [Loofbourow & Dwyer, 1939*a*, *b*], perhaps because of the effects of accumulated CO_2 on membrane permeability.

The present studies suggest that among the substances released by damaged cells into the intercellular *milieu*, various known growth factors and adenine nucleotides may account, in large part, for the effects of the suspension fluids on the metabolism of normal cells. The effects appear to be due to a variety of substances. This may explain the difficulties encountered in the application of chemical purification procedures alone to the elucidation of the problem—hence the emphasis now being placed on microbiological and chemical methods of analysis for known growth factors. From the standpoint of the possible relationship of the general phenomenon to wound healing and overgrowth, it might appear to be more profitable to concentrate upon studies of animal tissues. We believe, however, that a thorough study of the phenomenon with yeast, as a convenient and easily utilized organism, should point the way to the most direct approach to the problem of tissue repair in animals following cell damage.

The fact that, of the adenine complexes tested, adenosine triphosphate appeared more effective than the others in simulating the effects of damaged-cell products should not be interpreted as too strong an indication of the identity of this substance with the nucleotide moiety of the damaged-cell products. The stability of the damaged-cell preparations was greater than might be expected were adenosine triphosphate an essential constituent. Furthermore, the adenosine triphosphate preparation very likely contained an appreciable proportion of the di- and monophosphates. The question of the identity of the nucleotide moiety is being investigated in greater detail.

Summary

The proliferation promoting effect of products from damaged cells appears to be due to a variety of substances, among which are known growth factors and adenine nucleotides. Damaged-cell products caused increased growth of yeast when added to media well supplemented with biotin, pantothenic acid, folic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, choline, and amino-acids. Addition of adenosine triphosphate produced the same effect as addition of the damaged cell products. Adenosine triphosphate, muscle adenylic acid, and adenosine, but not yeast adenylic acid or yeast nucleic acid, increased the growth rate of yeast in synthetic media, with or without the addition of growth factor and amino-acid supplements. It appears, therefore, that certain adenine nucleotides act as growth factors. The metabolic effects of damaged-cell products were reproduced by a combination of known growth factors and adenosine triphosphate.

I wish to express my thanks to Dr Fritz Lippmann of the Harvard Medical School for supplying the preparations of adenosine triphosphate, muscle adenylic acid, and adenosine, to Mr Robert Sinsheimer for obtaining the data on solvent fractionations and to Mrs R. K. Abramowitz for assistance in the growth assays.

REFERENCES

Bishop, L. R. & Rainbow, C. [1939]. *J. Inst. Brew.* **45**, 33.
Cook, E. S. [1942]. Personal communication and *Stud. Inst. Divi Thomae*, in publication.
Cook, E. S., Hart, M. J. & Stimson, M. M. [1940]. *Biochem. J.* **34**, 1580.
Cook, E. S., Loofbourow, J. R. & Stimson, M. M. [1939]. *Atti X° Congr. Intern. Chem.* **5**, 26.
Fardon, J. C., Carrol, M. J. & Ruddy, M. V. [1938]. *Stud. Inst. Divi Thomae*, **1**, 35.
Joyce, L. [1940]. Unpublished.
Levene, P. A. & Bass, L. W. [1931]. Nucleic Acids. Chemical Catalog. Co., N.Y.

Loofbourow, J. R. [1942*a*]. *Biochem. J.* **36**, 631.
—— [1942*b*]. *Nature, Lond.*, **150**, 349.
Loofbourow, J. R., Cook, E. S., Dwyer, C. M. & Hart, M. J. [1939]. *Nature, Lond.*, **144**, 553.
Loofbourow, J. R., Cueto, A. A. & Lane, M. M. [1939]. *Arch. exp. Zellforsch.* **22**, 607.
Loofbourow, J. R. & Dwyer, C. M. [1938]. *Science*, **88**, 191.
—— —— [1939*a*]. *Nature, Lond.*, **143**, 725.
—— —— [1939*b*]. *Stud. Inst. Divi. Thomae*, **2**, 155.
Loofbourow, J. R., Dwyer, C. M. & Cronin, A. G. [1941]. *Biochem. J.* **35**, 603.
Loofbourow, J. R., Dwyer, C. M. & Lane, M. M. [1940]. *Biochem. J.* **34**, 432.
Loofbourow, J. R., Dwyer, C. M. & Morgan, M. N. [1938]. *Stud. Inst. Divi Thomae*, **2**, 137.
Loofbourow, J. R., Webb, A. M., Loofbourow, D. G. & Abramowitz, R. K. [1942]. *Biochem. J.* **36**, 513.
Menkin, V. [1941]. *Cancer Res.* **1**, 548.
Reader, V. [1927]. *Biochem. J.* **21**, 901.
Sizer, I. J. & Loofbourow, J. R. [1942]. To be published.
Stimson, M. M., Aquinas, M. & Loofbourow, J. R. [1940]. Unpublished.
Williams, R. J., Eakin, R. E. & Snell, E. E. [1940]. *J. Amer. chem. Soc.* **62**, 1204.

85. Blood Esterases

By **Derek Richter** and **Phyllis Godby Croft**, *Central Pathological Laboratory* (*L.C.C.*) *and Mill Hill Emergency Hospital, London, N.W. 7*

(*Received 14 August 1942*)

It has recently been shown that there is a rise in the choline esterase activity of the serum in states of severe emotional tension [Richter & Lee, 1942 *a*, *b*]. In attempting to assess the physiological significance of this observation and apply it for clinical purposes it was necessary to have further information as to the precise nature of the esterases occurring in blood.

Apart from the choline esterase, it has long been known that human serum contains a 'serum lipase', which hydrolyses simple aliphatic esters and glycerides such as methyl butyrate and tributyrin [Rona & Bach, 1920]. The 'serum lipase' has been found also in a number of other animal species, but little is known of it apart from the observation that the name 'lipase' is probably a misnomer, since the enzyme has little or no action on the true fats and there is no evidence that it has anything to do with lipid metabolism [Cherry & Crandall, 1932].

Vahlquist [1935] attributed the hydrolysis of the aliphatic esters, glycerides and choline esters to one and the same enzyme, but this was disputed by Hall & Lucas [1937]. Stedman and his collaborators [1932; 1933; 1935; 1937] showed that the blood esterases behave differently in different species. They found that the serum choline esterase in certain species exerted a considerable hydrolytic action on tributyrin, and a slight action on methyl butyrate; but the evidence for their conclusions was criticized by Shaw [1935].

The considerable divergences of opinion as to the specificity and other properties of the esterases in serum may be due in part to the different sources of material and the variety of experimental conditions used by different investigators. Certain factors, such as the effect of the neutral salt concentration and the inhibition of esterases by bromthymol-blue, which has frequently been used as an indicator in measuring esterase activities by the titration method, must also be considered as possible sources of error [Alles & Hawes, 1940; Vahlquist, 1935]. It is still not clear to what extent the esterases other than choline esterase are able to hydrolyse acetylcholine. It is also uncertain to what extent choline esterase is responsible for the hydrolysis of aliphatic esters and glycerides by serum and corpuscles in the different species which have commonly been used as experimental animals.

In view of these uncertainties a reinvestigation of the esterases occurring in blood has now been made with the object of defining and characterizing them more clearly and devising criteria for distinguishing between them that could be used, in particular, for purposes of clinical investigation.

Experimental methods

Material. Horse blood was collected under sterile conditions in glass flasks and brought immediately from the slaughterhouse to the laboratory. The human blood consisted of venous specimens taken from patients admitted on account of neurotic complaints, but in whom a careful examination had failed to show any significant physical abnormality. Cat, dog and rabbit blood were obtained without the use of anaesthetics from an artery or vein.

Esterase activity. Esterase activities were estimated manometrically with a Warburg apparatus by measuring the CO_2 liberated from bicarbonate buffer by the fatty acids formed by hydrolysis of the esters. The method was similar to that of Jones & Tod [1935] and no correction was applied for the CO_2 retained by buffers present. Serum (0·2 ml.) was diluted immediately before use to 4 ml. with a solution containing 0·03 *M* $NaHCO_3$ and 0·12 *M* NaCl which was kept in a closed bottle in equilibrium with 5 % CO_2 in O_2. The diluted serum was placed in the Warburg cup and the substrate in the side tube. The bath temperature was 37° and the vessels were filled with O_2 containing 5 % CO_2. The substrate for choline esterase was 0·5 ml. of a solution of 2·5 % acetylcholine chloride in 0·9 % NaCl. Methyl butyrate (0·1 ml.) and tributyrin (0·1 ml.) were used as substrates for the other esterases. Since these two esters are only sparingly soluble in water a slight excess of substrate remained undissolved. Preliminary experiments showed that under these conditions the hydrolysis-time curves gave satisfactorily straight lines during the first 30–40 min., and the enzyme activities, which were calculated from the slope, were reproducible to within about 10 %. When eserine or other solutions were used, 0·35 ml. of the required concentration was added to 2·65 ml. of diluted serum. Inhibitors were allowed to stand for 20 min. with the enzyme before adding the substrate. All additions were brought to *p*H 7·4 before use. Esterase activities are expressed as micro-equivalents (μE.) of ester hydrolysed per ml. of serum (or corpuscles) per hour. The abbreviations Ac.ch., Me.but. and Tribut. are used for acetylcholine, methyl butyrate and tributyrin respectively. The choline esterase activities (μE./ml./hr.) may be converted into the usual clinical units [Jones & Tod, 1935] by dividing by the factor 2·36.

Horse serum

Horse serum was examined in the first place since it has received considerable attention from other investigators and it is better understood than the other blood esterase systems. Stedman, Stedman & Easson [1932] and Stedman & Stedman [1935] found that horse serum contains two enzymes, (*a*) an esterase which hydrolyses simple esters such as methyl butyrate or tributyrin, and (*b*) a choline esterase which they believed to be entirely specific for choline esters, though Easson & Stedman [1937] concluded later that this was uncertain and that it might also exercise a small activity on other esters. Shaw [1935] criticized the evidence obtained by Stedman *et al.* [1932] and concluded that 'the esterase prepared from horse serum by the method of Stedman *et al.* [1932] is not choline esterase'. Easson & Stedman [1937] have replied to this criticism. The possibility that a third enzyme, a lipase, may also be present in horse serum was suggested by Stedman, Stedman & White [1933].

In the present investigation the term 'ali-esterase' has been used to indicate an esterase of the type that acts preferentially on the simple aliphatic esters and glycerides.

Separation of esterases by methods of partial destruction. Specimens of horse serum were tested with acetylcholine, methyl butyrate and tributyrin under the conditions described. They showed considerable activity with all three substrates:

Horse serum no.	Esterase activity in μE./ml./hr.		
	Ac.ch.	Me.but.	Tribut.
1	86	264	125
2	100	222	101

A number of procedures were applied to the serum to produce partial destruction of the enzymes: these consisted of (1) ageing by keeping for 14 days, (2) heating to 50–55°, (3) drying with acetone, (4) fractional precipitation with ammonium sulphate, and (5) treatment with adsorbents. When the resulting products were tested again with the three substrates, all these treatments except the fractional precipitation were found to

have produced a significant change in the relative activities towards the three substrates, indicating a partial separation into two enzymes. The resulting enzymic activities, expressed as a percentage of the original activity, are given in Table 1.

Table 1. *Partial destruction of esterases of horse serum*

Serum no.	Treatment	% of original activity		
		Ac.ch.	Me.but.	Tribut.
1	Ageing, 14 days	60	95	100
2	Ageing, 19 days	63	83	98
1	Deminrolit A at *p*H 4	81	12	19
2	Deminrolit A at *p*H 4	100	41	42
1	Fuller's earth at *p*H 4	22	96	56
2	Fuller's earth at *p*H 4	57	93	77
1	Heat 10 min. at 55°	67	11	17
2	Heat 30 min. at 50°	47	34	12
2	Acetone drying	2	6	7
1	$(NH_4)_2SO_4$ precip.	30	31	33
2	$(NH_4)_2SO_4$ precip.	19	18	15

Fractional precipitation by $(NH_4)_2SO_4$ was done exactly according to the directions given by Stedman *et al.* [1932]. Deminrolit A is a specific adsorbent prepared by the Permutit Co.; it was used after preliminary treatment with a small amount of fuller's earth. The serum was shaken with the adsorbents which were then removed by centrifuging.

The activity towards methyl butyrate and tributyrin generally underwent similar changes as a result of the various treatments, indicating that these two substrates were hydrolysed by the same enzyme, an ali-esterase distinct from the choline esterase. The choline esterase was much more resistant to destruction by heating, but less resistant to the effects of acetone drying, than the ali-esterase.

Inhibition by eserine. Eserine sulphate (10^{-5}) produced complete inhibition of the activity towards acetylcholine; but no significant inhibition with methyl butyrate. With tributyrin as substrate there was an inhibition of about 10%. This result was repeated and confirmed many times.

Serum no.	Ac.ch.		Me.but.		Tribut.	
	Alone	Eserine	Alone	Eserine	Alone	Eserine
1	86	0	256	242	106	95
2	107	0	182	186	96	81

It was clear that the ali-esterase had no action on acetylcholine, and conversely that the choline esterase of horse serum appeared to be almost entirely specific in that it showed no measurable activity towards methyl butyrate and only very slight activity towards tributyrin. These results confirm and extend the observations made by the Stedmans and their collaborators and agree entirely with their conclusions.

Human serum

Preliminary experiments with human serum showed that it readily hydrolysed the three esters used in the present study, but it differed from horse serum in that it was relatively more active towards acetylcholine and less active with methyl butyrate. The choline esterase activity varied greatly in different individuals, ranging in the present series from 67 to 279, and it was noticeable that the activity towards methyl butyrate and tributyrin generally varied in parallel with the choline esterase activity. This was shown by the ratios of the activities Ac.ch./Me.but. and Ac.ch./Tribut. Experiments with oxalated plasma gave results identical with those with serum (Table 2).

There was no reason to expect that two dissimilar enzymes such as an ali-esterase and a choline esterase should occur together in such relatively constant proportions. The probability of these figures occurring by chance was found on statistical analysis to be

Table 2. *Esterase activities of different human sera*

No.	Esterase activity in μE./ml./hr.			Relative activities	
	Ac.ch.	Me.but.	Tribut.	Ac.ch./Me.but.	Ac.ch./Tribut.
1	165	44	53	3·7	3·2
2	240	110	59	2·2	4·2
3	67	24	41	2·7	1·6
4	199	97	116	2·0	1·7
5	279	99	207	2·8	1·3
6	234	105	139	2·2	1·7
7	234	103	154	2·3	1·5
8	67	31	52	2·2	1·3
9	90	40	66	2·2	1·4
10	97	49	75	2·0	1·3
11	56	25	36	2·2	1·6
12	123	67	101	1·8	1·2
13	94	41	78	2·3	1·2
14	143	51	91	2·8	1·6
15	98	47	99	2·1	1·0
16	69	26	44	2·6	1·6

Oxalated plasma was used instead of serum in no. 16. The coefficient of correlation between the activities towards Ac.ch. and Me.but. calculated by the formula $\frac{\Sigma xy}{\sqrt{(\Sigma x^2 \times \Sigma y^2)}}$ [Fisher, 1938] was 0·94 and the probable error of the coefficient was 0·02. The correlation was therefore statistically significant.

very small since the correlation coefficient (0·94) was very high and greatly exceeded the probable error (0·02). This gave evidence that the enzymes hydrolysing the three substrates were either closely related or identical.

Attempted differentiation between enzymes. Human serum differed markedly from horse serum in that the same methods which were successful in achieving a partial separation into two enzymes in the horse failed completely with human serum.

The choline esterase of human serum showed little falling off in activity on keeping for periods of a few weeks, but an old specimen of plasma which had been kept for 18 months and still retained about half of the original activity showed no significant change from the normal ratio of activities:

Ac.ch.	Me.but.	Tribut.	Ac.ch./Me.but.	Ac.ch./Tribut.
69	26	44	2·6	1·6

The percentages of the original activities that remained after (1) heating to 55° for 20 min. and (2) treatment with adsorbents were as follows:

Treatment	% of original activity		
	Ac.ch.	Me.but.	Tribut.
Heat 20 min. at 55°	37	37	34
Adsorption on deminrolit A at *p*H 4	40	49	50

Inhibition by eserine. Eserine concentrations higher than about 10^{-6} caused almost complete inhibition of the hydrolysis of all three substrates by human serum, indicating again that they were probably all hydrolysed by the same enzyme, choline esterase.

Serum no.	Ac.ch.		Me.but.		Tribut.	
	Alone	Eserine, 10^{-5}	Alone	Eserine, 10^{-5}	Alone	Eserine, 10^{-5}
1	157	0	71	6	79	6
2	173	5	48	6	43	4
3	173	0	64	0	104	0
4	205	0	85	0	118	0
5	175	0	63	0	103	0

The difference between the systems in human and horse sera in this respect was brought out very strikingly by plotting the inhibition percentage against the eserine

concentration (Fig. 1). All esterase activity was strongly inhibited at very high eserine concentrations, but at intermediate concentrations of about 10^{-5} the horse-serum activity was only slightly inhibited towards methyl butyrate, while the inhibition with human serum was practically complete.

The results with human serum were consistent with the view that one enzyme, a choline esterase, was mainly responsible for the hydrolysis of all three substrates, but the slight irregularities in the Ac.ch./Me.but. and Ac.ch./Tribut. ratios in different subjects

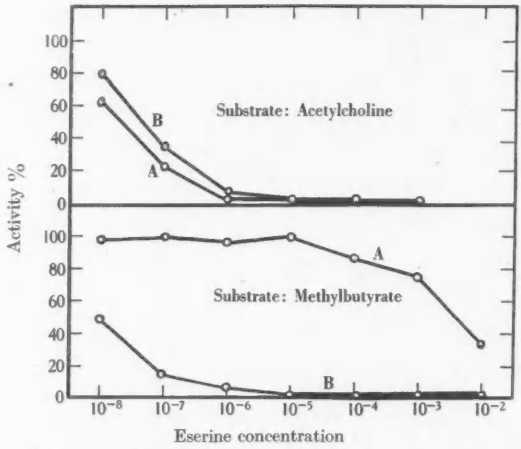


Fig. 1. Inhibition of esterases of horse serum (curves *A*) and human serum (curves *B*) by eserine. Activities expressed as percentage of activity without eserine.

suggested that a relatively small and irregular amount of a second ali-esterase may also occur. These observations agree closely with the conclusions of Vahlquist [1935] and Easson & Stedman [1937], but they disagree with those of Hall & Lucas [1937].

Human red blood corpuscles

Red blood corpuscles which had been washed free from serum with 0·9 % NaCl showed a greater activity than the serum towards acetylcholine and less activity towards tributyrin and methyl butyrate (Table 3).

Table 3. *Human red blood corpuscles*

	Esterase activity in μE./ml./hr.			Relative activities	
No.	Ac.ch.	Me.but.	Tribut.	Ac.ch./Me.but.	Ac.ch./Tribut.
1	156	65	56	2·4	2·8
2	134	43	32	3·1	4·2
3	240	39	54	6·1	4·4
4	348	44	61	7·9	5·7
5	310	58	148	5·3	2·1
6	350	60	91	5·8	3·8
7	360	68	86	5·3	4·2
8	350	65	84	5·4	4·2
9	280	38	61	7·4	4·6
10	370	34	50	10·9	7·4
11	339	128	81	2·6	4·2

The system differed from that in serum in that the activity towards acetylcholine and the aliphatic esters showed considerable independent variation. Treatment with fuller's

earth caused significant alterations in the relative activities towards the different substrates, which gave evidence of a partial separation into two enzymes:

	% of original activity		
Adsorbent	Ac.ch.	Me.but.	Tribut.
Fuller's earth	5	14	11

The presence of two or more esterases was confirmed by the behaviour towards 10^{-5} eserine, which caused complete inhibition of acetylcholine hydrolysis, but no significant inhibition of the hydrolysis of the other esters:

	Ac.ch.		Me.but.		Tribut.	
No.	Alone	Eserine, 10^{-5}	Alone	Eserine, 10^{-5}	Alone	Eserine, 10^{-5}
1	186	0	19	24	38	35
2	216	3	54	52	63	58
3	200	1	26	22	49	45

These experiments gave evidence of the existence in the red blood corpuscles of two enzymes, (*a*) an ali-esterase which hydrolysed simple aliphatic esters, and (*b*) a choline esterase which differed from the serum choline esterase in being completely specific in its action. Any activity of the choline esterase towards methyl butyrate or tributyrin was less than 3 % of the activity towards acetylcholine.

Cat blood

Using the inhibition by 10^{-5} eserine as a criterion for distinguishing between ali-esterases and choline esterases, the esterases occurring in cat serum and corpuscles were investigated (Table 4).

Table 4. *Differentiation between blood esterases by means of eserine*

			Activity in μE./ml. hr.		
Species	Tissue	Esterases	Ac.ch.	Me.but.	Tribut.
Horse	Serum	Choline esterase	107	0	11
		Ali-esterase	0	182	95
Man	Serum	Choline esterase	184	71	78
		Ali-esterase	0	0	0
	Corpuscles	Choline esterase	182	0	0
		Ali-esterase	0	22	45
Dog	Serum	Choline esterase	169	59	65
		Ali-esterase	0	12	6
	Corpuscles	Choline esterase	83	21	30
		Ali-esterase	0	26	24
Cat	Serum	Choline esterase	108	8	25
		Ali-esterase	8	36	90
	Corpuscles	Choline esterase	48	11	40
		Ali-esterase	0	27	69
Rabbit	Serum	Choline esterase	18	36	110
		Ali-esterase	0	97	300
	Corpuscles	Choline esterase	34	9	20
		Ali-esterase	0	83	130

The figures representing ali-esterase activities were obtained with the different substrates in the presence of 10^{-5} eserine: the choline esterase activities represent the additional activity found in the absence of eserine.

The serum choline esterase appeared to be unspecific for acetylcholine when examined in this way, since the activity of the serum towards the aliphatic esters was significantly inhibited by eserine. An ali-esterase was also present.

The corpuscle choline esterase was not specific for acetylcholine; the ali-esterase present in the corpuscles was less active than that in the serum.

Dog blood

With dog serum the hydrolysis of all three substrates was markedly inhibited by eserine, indicating that the serum choline esterase showed considerable activity towards the aliphatic esters. The ali-esterase showed no activity towards acetylcholine.

The esterases present in the corpuscles appeared to consist, as in the cat, of a non-specific choline esterase and an ali-esterase which did not hydrolyse acetylcholine.

Rabbit blood

In rabbit serum there appeared to be a relatively inactive choline esterase, and a very active ali-esterase which showed no activity towards acetylcholine. In the corpuscles two enzymes similar to those in the serum were found.

The figures given in Table 4 should be treated with considerable reserve. Activities of the order of 10 or less approached the experimental error and are therefore doubtful. It should be emphasized that the differentiation between the ali-esterases and choline esterase depended entirely on the assumption that the former was unaffected, while the latter was completely inhibited, by 10^{-5} eserine. This assumption was justified in the experiments with human and horse serum by the inhibition curves (Fig. 1); but in the tissues which showed relatively little choline esterase activity, such as rabbit serum, it may be doubted whether this still holds good. Apart from these limitations, the figures give a general indication of the approximate order of the esterase activities in the serum and corpuscles of a number of species, and they may be useful until more precise methods of differentiating between the different esterases have been worked out in each individual case. A specific inhibitor for the ali-esterase would be of great value for this purpose, but all attempts to find such an inhibitor have hitherto proved unsuccessful. It is possible that in some species more than one ali-esterase may be present.

Inhibition of esterases by quinine and atoxyl

Earlier investigators achieved some success in differentiating between different types of esterases by making use of quinine and atoxyl as inhibitors. Liver esterase, for example, is stated to be inhibited by atoxyl but not by quinine, while 'serum lipase' is inhibited by both [Rona & Bach, 1920; Petow, 1929].

A series of blood systems was now examined by means of these inhibitors to test how far they might be useful for the purpose of the present investigation and to bring the present and earlier observations into line. The inhibitor concentrations were similar to those used by Petow [1929] in the clinical examination of esterases in tissues. The conditions were otherwise the same as have been previously described (Table 5).

Table 5. *Inhibition and acceleration of esterases by atoxyl and quinine*

Species	Tissue	Inhibitor	Substrate: Ac.ch.	Substrate: Me.but.	Species	Tissue	Inhibitor	Substrate: Ac.ch.	Substrate: Me.but.
Horse	Serum	Atoxyl	100	100	Cat	Serum	Atoxyl	100	90
		Quinine	21	100			Quinine	32	130
Man	Serum	Atoxyl	53	100		Corpuscles	Atoxyl	120	109
		Quinine	23	228			Quinine	90	130
	Corpuscles	Atoxyl	100	100	Rabbit	Serum	Atoxyl	100	100
		Quinine	70	100			Quinine	53	32
Dog	Serum	Atoxyl	100	100		Corpuscles	Atoxyl	100	120
		Quinine	34	165			Quinine	69	74
	Corpuscles	Atoxyl	167	92					
		Quinine	67	185					

All activities are expressed as percentage of the activity with substrate alone. Concentration of quinine sulphate 0·2 mg./ml. Concentration of atoxyl 0·02 mg./ml.

The most striking result of these experiments was in showing that the behaviour of the choline esterases and ali-esterases towards the two inhibitors depended greatly on the source from which they were derived. In some cases strong inhibitions were found, in others there was no apparent effect, while in a few instances accelerations were observed.

Quinine produced in every case a partial inhibition of the hydrolysis of acetylcholine by choline esterase; but in several cases a marked acceleration with methyl butyrate as substrate was observed.

The accelerating action of quinine on the hydrolysis of methyl butyrate by human serum was previously reported by Vahlquist [1935]. In the present investigation atoxyl was also found to exert an accelerating effect on the hydrolysis of acetylcholine by the choline esterase of dog and cat corpuscles. That the choline esterase, and not the ali-esterase, was concerned in these accelerations was shown by the inhibition obtained on the further addition of 10^{-5} eserine.

	Me.but.			Ac.ch.		
	Alone	Quinine	Quinine + 10^{-5} eserine	Alone	Atoxyl	Atoxyl + 10^{-5} eserine
Human serum	100	228	15	—	—	—
Cat corpuscles	100	130	50	100	120	0
Dog serum	100	165	18	—	—	—
Dog corpuscles	—	—	—	100	167	0

Activities expressed as percentage of activity with substrate alone.

Sulphanilic acid, which resembles atoxyl in molecular configuration, produced no effect when added in the same concentration as atoxyl, in the hydrolysis of acetylcholine by the choline esterase of dog blood corpuscles.

The effects of the various inhibitors and accelerators depended on the substrate used; for example, quinine accelerated with methyl butyrate but inhibited the hydrolysis of tributyrin by choline esterase.

Inhibition by amines

The accelerating action of quinine on the hydrolysis of methyl butyrate by choline esterases raised the question of whether this is a special property of quinine or whether it is a general property of organic bases. The effect of a series of amines on the hydrolysis of methyl butyrate by human and horse serum was therefore tested.

	Human serum	Horse serum
Triethylamine	84	88
Ethylamine	81	85
Methylamine	94	77
Choline	92	97

Amine concentration: 2·5 mg./ml. Activities expressed as percentage of activity with substrate alone.

In every case an inhibition was observed.

Hydrolysis of castor oil by blood esterases

Orientating experiments were carried out to test the lipolytic activity of the serum and corpuscles in man, horse and dog, using castor oil as a substrate, with and without the addition of bile salt as a lipase activator.

A 10% emulsion (0·5 ml.) of castor oil and water prepared as described by Cole [1933] was placed in the side tube of the Warburg vessel; the main vessel contained 1·5 ml. of serum or corpuscles diluted with 1·5 ml. of bicarbonate buffer. When bile salt was used, 0·4 ml. of a solution containing 50 mg. sodium tauroglycocholate per ml. was added to the main vessel and 1·1 ml. of buffer was used. Some slight activity was noted in the experiments with dog serum; after the addition of bile salt dog corpuscles also showed slight activity, but in general there was little activity and in no case did the activity exceed 5 μE./ml./hr. It was clear that none of the tissues contained a lipase comparable in activity with the ali-esterases and choline esterases that have been described.

Hydrolysis of cholesterol acetate by blood esterases

The hydrolysis of cholesterol acetate by sera and corpuscles was tested under the same conditions as in the experiments with castor oil, with and without the addition of bile salt. The substrate was used as an emulsion which was prepared by shaking an acetone solution containing 50 mg./ml. cholesterol acetate with 30 ml. water and then dialysing the emulsion against water to remove the acetone.

The greatest activity was found with dog corpuscles which, in the presence of bile salt, gave an activity of 12 μE./ml./hr., but none of the tissues showed an activity greater than 1 μE./ml./hr. in the absence of bile salt.

The sterol esterase activity in these tissues was therefore relatively small.

Discussion and Conclusions

The esterase activities of the sera and red blood corpuscles in a number of species have been measured by the manometric method in the presence of NaCl and $NaHCO_3$-CO_2 buffer at 37°. By the use of different substrates, specific inhibitors and various methods of separation, data were obtained as to the specificities and other properties of the esterases occurring in the different tissues. The results differed to some extent from those obtained by other investigators; but they are in general agreement with the main conclusions of the Stedmans, particularly, and they confirm many of their results.

Esterases occurring in blood

Choline esterases. From a physiological point of view choline esterases are characterized chiefly by (*a*) their ability to hydrolyse choline esters, and (*b*) their inhibition by eserine. Eserine also inhibits other esterases at high concentrations, and this is responsible for some confusion in the literature [Ammon, 1935], but at a concentration of 10^{-5} eserine is highly specific as a choline esterase inhibitor. The two criteria of hydrolysing choline esters and eserine inhibition were sufficiently definite to allow the choline esterases to be distinguished sharply from the other esterases occurring in blood. The choline esterases obtained from certain sources appeared to be entirely specific in that they showed no measurable activity towards methyl butyrate or tributyrin; choline esterases obtained from other sources were not specific. The activity of human serum choline esterase, for example, towards methyl butyrate and tributyrin was about 50 % of the activity towards acetylcholine, and the activity towards methyl butyrate could be further increased by the addition of quinine.

The choline esterase of human red blood corpuscles contrasted strongly with serum choline esterase in being far more specific towards acetylcholine. It showed no measurable activity towards methyl butyrate or tributyrin, and under the conditions described any activity towards these substrates was less than 3 % of the activity towards acetylcholine. It showed no acceleration with quinine.

Previous investigators have frequently referred to choline esterase as if it were one specific enzyme, but in the present investigation we have been impressed by the great differences in specificity and other properties of the choline esterases obtained from different sources. This was also observed in certain instances by Alles & Hawes [1940] and by Glick [1941]. It would appear more accurate to describe the choline esterases as a group of enzymes showing considerably divergent properties, rather than to regard choline esterase as a single entity. It is also clear that attempts which have been made to deduce the physiological behaviour of the choline esterases in muscle and nervous tissue from studies of the choline esterases of serum are liable to error on this account.

Ali-esterases. The word 'esterase' is commonly used as a comprehensive term to include the enzymes of all types that hydrolyse esters. The term 'ali-esterase' has been used in

the present work to describe the particular type of esterase that acts preferentially on the simple aliphatic esters and glycerides. The ali-esterases in blood had little or no action on fats, acetylcholine, or cholesterol acetate under the conditions of the present investigation.

Ali-esterases were found in the sera and red blood corpuscles in several species, but they were absent or present in only very small amounts in human serum. The ali-esterases of blood were similar in substrate specificity and in inhibition by surface-active substances to the well-known liver esterase, but they showed characteristic differences in their inhibition by atoxyl and quinine.

Lipases. There is evidence that the leucocytes contain a small amount of a true lipase that hydrolyses fats [Kraut & Burger, 1938]; but orientating experiments, using castor oil under the conditions described, confirmed the conclusion of other investigators that there is normally little or no true lipase present in the serum. Lipases may enter the serum from the pancreas or other organs in pathological conditions: the properties of the lipase then depend on the organ in which it originated, and in some cases this may be inferred from its inhibition by quinine or atoxyl [Petow, 1929; Cherry & Crandall, 1932].

Sterol esterases. Esterases which hydrolyse the esters of cholesterol and other sterols have been described in the serum in several species [Schramm & Wolff, 1940]: they are stated to be distinct from the other esterases which have been described. Preliminary experiments showed that they are very much less active than the ali-esterases or choline esterases and they were therefore not further investigated.

Esterases in the blood in different species

Figures giving the approximate order of activity of the blood esterases in a number of species are summarized in Table 4.

Horse serum. The existence of two esterases, a choline esterase and an ali-esterase, in horse serum was confirmed in a number of different experiments. The choline esterase did not act to any appreciable extent on methyl butyrate, but there was evidence that it acted slowly on tributyrin under the conditions of our experiments.

Human serum. Human serum showed a considerable activity towards methyl butyrate and tributyrin as well as towards acetylcholine. The apparent divergence of our figures from those of Easson & Stedman [1937] in this respect was probably due to the different experimental conditions. A variety of experiments designed to effect a separation into two or more enzymes, including (*a*) heat treatment, (*b*) partial destruction by ageing, and (*c*) treatment with adsorbents, agreed with the results of (*d*) experiments with inhibitors and (*e*) the measurement of the relative activities in different sera, in indicating that one enzyme only is mainly responsible for the activity towards all three substrates. This is not in agreement with the views of Hall & Lucas [1937], but it agrees with Vahlquist [1935] and Easson & Stedman [1937].

It is concluded that there is no evidence for the existence of a 'serum lipase' in normal human serum. Pancreatic lipase, or lipase from other organs, may occasionally occur in pathological sera, but normal serum contains little or no true lipase. Human serum may contain a small and variable amount of an ali-esterase derived from the red blood corpuscles [Richter & Croft, 1942], but the activity of normal human serum towards methyl butyrate and tributyrin is due mainly to the serum choline esterase, which shows considerable activity towards simple aliphatic esters and glycerides. The term 'serum lipase' is misleading and should be abandoned.

The choline esterase of human serum, like other choline esterases, was inhibited by a variety of organic bases; but the activity towards methyl butyrate was characteristically increased by over 100 % by the addition of a small amount of quinine. This accelerating action was not shown with acetylcholine or tributyrin as substrates, or with simpler bases such a triethylamine in the place of quinine.

The activity of human serum choline esterase varied considerably from one individual to another. Previous work has shown that the activity is lowered in conditions of debility and increased in acute emotional states and in other conditions associated with increased activity of the autonomic nervous system [Richter & Lee, 1942 *a*, *b*]. A temporary rise in activity is produced as a result of vigorous exercise [Richter & Croft, 1942].

Human red blood corpuscles. The esterases in blood corpuscles are of particular interest from a clinical point of view, owing to the possibility of their getting into the serum through partial haemolysis in vitro, or as has been observed in conditions such as pernicious anaemia, through haemolysis in vivo [Simon, 1924].

Using the methods that proved most effective in the case of horse serum, namely, (*a*) comparison with three substrates, (*b*) inhibition by 10^{-5} eserine and (*c*) fractional adsorption, it was found that the red blood corpuscles contained two esterases, (*a*) a choline esterase, and (*b*) an ali-esterase. The choline esterase was much more active than the serum choline esterase and differed from it strikingly in that it was highly specific and showed no measurable activity towards methyl butyrate or tributyrin. It also showed no acceleration with quinine. The ali-esterase was relatively inactive: it was not appreciably inhibited by atoxyl.

Previous investigators have considered the possibility of there being a definite distribution of choline esterase between the blood plasma and cells [Stedman & Russell, 1937; Sabine, 1940], but it was found by Richter & Lee [1942*a*] that the serum choline esterase does not come from the corpuscles. The differences in specificity and in the behaviour of these two enzymes towards atoxyl and quinine make the possibility of a distribution between the plasma and corpuscles very improbable.

Cat, dog and rabbit blood. In cat, dog and rabbit blood a choline esterase and an ali-esterase were present in both the serum and the corpuscles. The choline esterase activity of rabbit blood was relatively low, but cat and rabbit blood contained very active ali-esterases in both serum and corpuscles.

Summary

1. The esterase activities of the blood corpuscles and sera in horse, man, dog, cat and rabbit have been estimated manometrically in the presence of NaCl and $NaHCO_3$ at 37°.

2. The properties of the choline esterases and 'ali-esterases' (i.e. those attacking simple aliphatic esters) occurring in these tissues have been examined by the use of specific inhibitors and methods of partial separation.

3. The choline esterases occurring in different species, and in serum and corpuscles of the same species, differ greatly in their properties.

4. The hydrolysis of methyl butyrate and tributyrin by human serum is mainly accounted for by the choline esterase. There is no evidence for the existence of a 'serum lipase' in normal human serum.

5. The choline esterase of human red blood corpuscles differs from the serum choline esterase in being entirely specific for choline esters and in behaving differently towards inhibitors.

6. Quinine inhibits the action of choline esterases on acetylcholine, but it accelerates the action of choline esterases derived from certain sources on methyl butyrate.

7. The hydrolysis of acetylcholine by the choline esterase of dog and cat corpuscles is accelerated by atoxyl.

8. The ali-esterases in blood show no activity towards acetylcholine.

The authors thank Dr Maclay and the Clinical Staff of the Hospital for their generous co-operation and the Rockefeller Foundation for supporting this investigation.

REFERENCES

Alles, G. A. & Hawes, R. C. [1940]. *J. biol. Chem.* **133**, 375.
Ammon, R. [1935]. *Ergebn. Enzymforsch.* **4**, 102.
Cherry, J. S. & Crandall, L. A. [1932]. *Amer. J. Physiol.* **100**, 266.
Cole, S. W. [1933]. Practical Physiological Chemistry, p. 184. Cambridge: Heffer.
Easson, L. H. & Stedman, E. [1937]. *Biochem. J.* **31**, 1723.
Fisher, R. A. [1938]. Statistical Methods for Research Workers, p. 128. Edinburgh: Oliver and Boyd.
Glick, D. [1941]. *Nature, Lond.*, **148**, 662.
Hall, G. E. & Lucas, C. C. [1937]. *J. Pharmacol.* **61**, 10.
Jones, M. S. & Tod, H. [1935]. *Biochem. J.* **29**, 2242.
Kraut, H. & Burger, H. [1938]. *Hoppe-Seyl. Z.* **253**, 105.
Petow, H. [1929]. Oppenheimer's Die Fermente und ihre Wirkungen, **3**, 1502.
Richter, D. and Croft, P. G. [1942]. In the Press.
Richter, D. & Lee, M. H. [1942*a*]. *J. ment. Sci.* **88**, 428.
—— —— [1942*b*]. *J. ment. Sci.* **88**, 435.
Rona, P. & Bach, E. [1920]. *Biochem. Z.* **111**, 166.
Sabine, J. C. [1940]. *J. clin. Invest.* **19**, 833.
Schramm, G. & Wolff, A. [1940]. *Hoppe-Seyl. Z.* **263**, 73.
Shaw, F. H. [1935]. *Aust. J. exp. Biol. med. Sci.* **13**, 251.
Simon, H. [1924]. *Z. ges. exp. Med.* **39**, 407.
Stedman, E. & Russell, W. R. [1937]. *Biochem. J.* **31**, 1987.
Stedman, E. & Stedman, E. [1935]. *Biochem. J.* **29**, 2107.
Stedman, E., Stedman, E. & Easson, L. H. [1932]. *Biochem. J.* **26**, 2056.
Stedman, E., Stedman, E. & White, A. C. [1933]. *Biochem. J.* **27**, 1055.
Vahlquist, B. [1935]. *Skand. Arch. Physiol.* **72**, 133.

86. Urea Formation in Mammalian Liver

By **H. A. Krebs**, *Department of Biochemistry, University of Sheffield*

(*Received 11 August 1942*)

Leuthardt [1938*a*] and Bach [1939] have expressed the opinion that in addition to the ornithine cycle there are other mechanisms of urea synthesis in the mammalian liver. Leuthardt [1938*a*] found that liver of starved guinea-pigs forms urea more rapidly from glutamine than from NH_4Cl. He concluded that NH_3 cannot be an intermediate in the urea formation from glutamine and that there is a more or less direct conversion of glutamine into urea. He suggested the following scheme:

$$\text{Glutamine} + 2HCO_3^- = \text{urea} + \text{pyrollidone carboxylic acid.} \qquad (1)$$

This scheme, however, is not in accordance with the fact that in liver tissue glutamine yields not pyrollidone carboxylic acid but glutamic acid [Krebs, 1935*b*], and Leuthardt [1938*b*] later appears to have given up the above scheme in favour of the more general idea that glutamine might act as a direct nitrogen donator to the 'urea forming system'.

Bach [1939] proposed two further mechanisms of urea formation, viz. 'urea synthesis from citrulline by oxidative hydrolysis':

$$\text{Citrulline} + H_2O + O \rightarrow \text{urea} + \text{glutamic acid},$$

and 'the oxidative conversion of ornithine and citrulline into glutamic acid and glutamine, where the pathway joins that suggested by Leuthardt':

$$\text{Ornithine} \xrightarrow{+O} \text{glutamine} \xrightarrow{+CO_2+NH_3} \text{urea} + \text{glutamic acid}.$$

In support of these schemes Bach quotes experiments which in his view demonstrate that α-ketoglutarate and pyruvate increase the rate of urea formation from citrulline and from ornithine and NH_3. He assumed that the ketonic acids acted directly or indirectly as agents oxidizing citrulline and ornithine.

Trowell [1942] recently studied the urea formation in perfused rat liver. Although his experiments confirmed the catalytic effect of ornithine on the synthesis of urea, and also the urea formation from citrulline and NH_3, Trowell came to the conclusion that 'the normal process of urea formation from ammonia is still obscure' and that 'it is clear... that the theory of an ornithine cycle is untenable'.

The present paper is chiefly a critique of the experimental findings and of the conclusions of the above authors.

Methods

The rate of urea formation in liver slices was measured as described by Krebs & Henseleit [1932*c*]. The only difference from the previous procedure was a modification of the method of urea determination. Aniline (0·1 ml.) and 3*M* acetate buffer (1 ml.) were added to the unknown solution. An extract of Arlco jack-bean meal (0·5 ml.) was used as urease. The object of adding aniline was to decompose the acetoacetic acid which is always formed in significant quantities together with urea when liver slices are incubated with ammonium salts [see Edson, 1935]. The slow decomposition of acetoacetic acid into acetone and CO_2 interferes with the urea determination. With the old procedure it was therefore sometimes necessary to wait 40 or 60 min. for completion of the CO_2 evolution from acetoacetic acid before the urease could be added. In the presence of aniline the decomposition of acetoacetic acid is complete within the ordinary 20 min. period of thermal equilibration.

In some experiments *l*(+)arginine was determined by enzymic conversion into urea and ornithine and manometric determination of the urea, a principle used before by Weil & Russell [1934], Baldwin [1935] and Hunter & Pettigrew [1937]. To obtain a solution of arginase, fresh mammalian liver (rat, rabbit, mouse or guinea-pig) was ground with sand and 3 vol. 0·01 *M* $NaHCO_3$ solution. The suspension was centrifuged and the supernatant was used as 'arginase'. A drop of octyl alcohol was added as a preservative. The extract keeps in the refrigerator for about one month.

The solution in which arginine was to be determined (3–6 ml.) was neutralized against litmus (if necessary) and enough *M* $NaHCO_3$ was added to make the final bicarbonate concentration 0·04 *M*. After addition of 0·25 ml. 'arginase' the solution was incubated for 60 min. at 40°. This was sufficient for the hydrolysis of 5 mg. arginine. The urea formed was determined as described above. In general, two controls were required. The first measured the urea and arginine content of the 'arginase'; it was set up together with the arginine determination, the unknown solution having been replaced by water. The second control measured the urea content of the unknown solution, an aliquot of which was tested. The urea value obtained in the controls was deducted from the urea in the unknown solution.

The hydrolysis of 174 mg. (anhydrous) arginine yields 60 mg. urea and subsequently 22,260μl. CO_2. Hence 1μl. CO_2 is equivalent to 0·00782 mg. arginine. The method thus yields reasonably accurate results if quantities from 0·1 mg. upwards are available.

This method shows a high degree of specificity. There are a few compounds related to *l*(+)arginine which can be hydrolysed by arginase, but these are unlikely to be present; moreover the rate of hydrolysis is in these cases exceedingly slow, viz. about 0·1–1% of the rate of the hydrolysis of *l*(+)arginine. This applies to *d*(−)arginine [Edlbacher & Zeller, 1936], arginylarginine [Edlbacher & Bonem, 1925; Edlbacher & Burchard, 1931], *l*(+)N-monobenzoyl-arginine [Felix, Müller & Dirr, 1928], *l*(+)arginic acid [Calvery & Block, 1934; Felix & Müller, 1936; Hunter & Woodward, 1941], (+)octopine [Akasi, 1937], α-N-methyl arginine, glycocyamine, ε-guanidino-α-amino-caproic acid [Felix & Schneider, 1938], carbamido-arginine [Hunter, 1938], δ-guanidino-valeric acid [Hellermann & Stock, 1938], and canavanine [Damodaran & Narayanan, 1940]. If any of these compounds is likely to be present it will be useful to follow the course of the urea production on addition of arginase. Four samples of the solution are incubated with arginase and urea is determined after 30, 60, 120 and 240 min. If the urea formation is practically completed after 30 min., *l*(+)arginine may be taken to be the only urea forming substance in the solution.

The crude liver extract used as arginase contained peptidases which liberate arginine from peptones (e.g. Bacto-peptone) and from peptides, and the arginine formed would therefore include 'bound' arginine. Proteins (casein, gelatine) do not yield arginine. 'Arginase' should not be prepared from frog and fish liver, for livers from these groups contain xanthine oxidase, uricase, allantoinase and allantoicase and therefore form urea from purine bases, uric acid, allantoin and allantoic acid.

Most experiments were carried out in duplicate. The temperature was 40°; the duration of the experiments was generally 60 or 90 min.

Glutamine and urea synthesis

Table 1 confirms Leuthardt's chief observation: in the liver of starved guinea-pigs (though not of well-fed guinea-pigs or of rats) the rate of urea formation from glutamine is 2–3 times greater than the rate of urea formation from ammonium *chloride*. On the other hand the rate of urea formation from ammonium *glutamate* is of the same order as that from glutamine and the facts are thus in accordance with the view that ammonium glutamate is an intermediate in the urea formation from glutamine:

$$\text{glutamine} \xrightarrow{\text{glutaminase}} \text{ammonium glutamate} \xrightarrow{\text{ornithine cycle}} \text{urea}.$$

It is therefore unnecessary to postulate a more or less direct conversion of glutamine into urea.

For the interpretation of the effects of glutamine and glutamate it is significant that ammonium lactate has a similar effect. A common property of these substances is the stimulation of liver respiration and this suggests that the effect on the urea synthesis may be indirect. As the urea synthesis depends on respiration, conditions which increase respiration may thereby also increase the rate of urea synthesis, although there is no simple numerical relationship between the two reactions [see Krebs, 1934].

Table 1. *Effect of glutamine and related substances on urea synthesis in liver*

'Glutamate' refers to *l*(+)glutamic acid, 'glutamine' to *l*(+)glutamine.

No.	Nutritional condition	Substances added to saline (final concentrations)	Q_{urea}
		Guinea-pig	
1	Starved 72 hr.	0·01 *M* NH_4Cl	1·83
		0·02 *M* glutamine	3·74
		0·02 *M* Na-glutamate; 0·01 *M* NH_4Cl	4·68
		0·02 *M* Na-α-ketoglutarate; 0·01 *M* NH_4Cl	2·20
		0·05 *M* glucose; 0·01 *M* NH_4Cl	1·79
		0·02 *M* *l*(+)Na-lactate; 0·01 *M* NH_4Cl	3·94
2	Starved 48 hr.	0·01 *M* NH_4Cl	2·23
		0·02 *M* glutamine	4·10
		0·02 *M* glutamine; 0·01 *M* NH_4Cl	4·38
		0·02 *M* Na-glutamate; 0·01 *M* NH_4Cl	4·10
		0·02 *M* Na-α-ketoglutarate; 0·01 *M* NH_4Cl	2·73
		0·02 *M* *l*(+)Na-lactate; 0·01 *M* NH_4Cl	4·45
3	Starved 65 hr.	0·01 *M* NH_4Cl	1·31
		0·02 *M* glutamine	4·23
		0·02 *M* glutamine; 0·01 *M* NH_4Cl	3·51
		0·02 *M* Na-glutamate; 0·01 *M* NH_4Cl	4·19
4	Starved 48 hr.	0·01 *M* glutamine	3·65
		0·01 *M* NH_4Cl	1·18
		0·02 *M* Na-glutamate; 0·002 *M* NH_4Cl	3·81
5	Starved 72 hr.	0·01 *M* NH_4Cl	1·05
		0·3 *M* glutamine	1·85
		0·3 *M* Na-glutamate; 0·01 *M* NH_4Cl	2·70
		0·3 *M* Na-succinate	0·86
		0·3 *M* Na-citrate	0·77
		0·3 *M* Na-fumarate	1·28
		0·3 *M* Na-α-ketoglutarate	0·86
		0·3 *M* *l*(+)lactate	2·09
6	Starved 48 hr.	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate	4·65
		0·01 *M* glutamine; 0·01 *M* *l*(+)lactate	3·87
7	Starved 72 hr.	0·01 *M* NH_4Cl	1·27
		0·02 *M* glutamine	4·67
		0·02 *M* Na-glutamate; 0·01 *M* NH_4Cl	3·20
8	Well fed	0·02 *M* glutamine	2·82
		0·01 *M* NH_4Cl	2·22
		0·02 *M* Na-glutamate; 0·01 *M* NH_4Cl	2·43
		Rat	
9	Starved 48 hr.	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate	6·12
		0·01 *M* glutamate; 0·01 *M* *l*(+)lactate	4·04
		0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate; 0·001 *M* *l*(+)ornithine	18·6
10	Well fed	0·01 *M* NH_4Cl	2·61
		0·03 *M* glutamine	0·88

An alternative explanation might be based on the recent discovery by Borsook & Dubnoff [1941] of a synthesis of arginine from citrulline and glutamic acid. This reaction occurs in the kidney of rat and guinea-pig, but has so far not been found in the liver [Borsook & Dubnoff, 1941]. It was thought possible that in the special case of the starved

guinea-pig liver the reaction might occur, and be the cause of Leuthardt's observations and this possibility was tested with the method for the determination of arginine described above. The experiments fully confirm Borsook & Dubnoff's findings with kidney cortex; a rapid synthesis of arginine from citrulline and glutamic acid occurs in this tissue (Table 2). In the liver the reaction of Borsook & Dubnoff is not expected to result in the

Table 2. *Synthesis of arginine from citrulline and* l(+)*glutamic acid in kidney cortex*

Period of incubation 90 min.; 3 ml. saline; 5% CO_2 in O_2; 40°;

$$Q_{(\text{urea}+\text{arginine})} = \frac{\text{urea found after incubation with arginase}}{\text{mg. tissue} \times \text{hr.}}.$$

Exp.	Species	Substrates	Q_{urea}	$Q_{(urea+arginine)}$
1	Rat	0·008 *M* citrulline		1·99
		0·008 *M* citrulline; 0·005 *M* glutamate		5·03
2	Rat	None	0·11	0·42
		0·005 *M* citrulline	0·17	1·40
		0·005 *M* glutamate	0·29	0·85
		0·015 *M* citrulline + 0·005 *M* glutamate	0·87	5·20
3	Rat	0·006 *M* citrulline		1·79
		0·006 *M* citrulline; 0·006 *M* glutamate		5·44
4	Guinea-pig	None	0·57	0·70
		0·01 *M* citrulline	0·53	0·53
		0·001 *M* glutamate	0·49	0·40
		0·01 *M* citrulline + 0·005 *M* glutamate	0·74	1·72
5	Guinea-pig	0·01 *M* citrulline	0·49	0·77
		0·01 *M* citrulline; 0·003 *M* glutamate	0·41	1·53

accumulation of arginine, because of the high arginase activity of the liver. Urea and ornithine would be found instead of arginine. Hence citrulline and glutamate together should yield more urea than citrulline alone if the reaction of Borsook & Dubnoff occurred. Table 3 shows that glutamate has some effect on the urea formation from citrulline but as glutamate, like lactate, accelerates the urea formation from citrulline and NH_4Cl

Table 3. *Urea formation from citrulline in guinea-pig liver*

Period of incubation 90 min.; 40°.

Exp.	State of nutrition	Substrates	Q_{urea}
1	Well fed	None	0·78
		0·01 *M* citrulline	1·28
		0·01 *M* citrulline; 0·01 *M* glutamate	1·37
		0·01 *M* glutamate	0·88
		0·01 *M* citrulline; 0·005 *M* aspartate	1·16
		0·01 *M* citrulline; 0·01 *M* glutamate; 0·005 *M* NH_4Cl	3·91
2	Well fed	None	0·51
		0·01 *M* citrulline	0·71
		0·01 *M* citrulline; 0·01 *M* glutamate	0·70
		0·01 *M* citrulline; 0·01 *M* NH_4Cl	2·44
		0·01 *M* NH_4Cl	2·21
3	Starved 48 hr.	None	1·01
		0·01 *M* citrulline	2·25
		0·01 *M* citrulline; 0·01 *M* glutamate	3·55
		0·01 *M* glutamate	1·38
		0·01 *M* NH_4Cl	0·70
		0·01 *M* NH_4Cl; 0·01 *M* citrulline	2·74
4	Starved 48 hr.	None	1·26
		0·01 *M* citrulline	1·85
		0·01 *M* glutamate	1·65
		0·01 *M* citrulline; 0·01 *M* glutamate	2·85
		0·01 *M* NH_4Cl	1·79
		0·01 *M* citrulline; 0·01 *M* NH_4Cl	1·91
		0·01 *M* glutamate; 0·01 *M* NH_4Cl	4·48
		0·01 *M* citrulline; 0·01 *M* glutamate; 0·01 *M* NH_4Cl	7·16

(see Exp. 4) these effects do not indicate that glutamate is a direct nitrogen donator. There is thus no reason for assuming that the reaction of Borsook & Dubnoff occurs in guinea-pig liver.

Rates of urea formation in the presence of ornithine and citrulline

Borsook & Dubnoff raise the question of the source of the citrulline required for the synthesis of arginine in the kidney and express doubt over the possibility that the liver can supply the citrulline, because in the ornithine cycle 'the existence of citrulline is only transitory on its way to arginine'. Experiments published previously [Krebs & Henseleit, 1932*c*] indicated that the rates of urea formation in the presence of ornithine and citrulline are of the same order of magnitude and this result is confirmed by further experiments recorded in Table 4. In Exp. 2, Q_{urea} is a little greater in the presence of citrulline and in this case the synthesis of citrulline is probably the reaction limiting the rate of the cycle. In the other experiments the rates are virtually identical with ornithine and citrulline and here the conversion of citrulline into arginine is probably the limiting reaction. In the latter case, an accumulation of citrulline in the solution may be expected, and actually occurs as shown by Gornall & Hunter [1941]. In vivo the excess of citrulline will enter the blood circulation and thus reach the kidney. There is thus no difficulty in assuming that the liver is the source of the citrulline in the reaction of Borsook & Dubnoff.

Table 4. *Formation of urea in the presence of ornithine and of citrulline, in rat liver*

Exp.	Substrates added (final concentrations)	Q_{urea}
1	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate; 0·01 *M* *l*(+)ornithine	9·0
	,, ,, 0·01 *M* *l*(+)citrulline	9·2
2	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate; 0·005 *M* *l*(+)ornithine	8·7
	,, ,, 0·005 *M* *l*(+)citrulline	10·9
3	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate; 0·005 *M* *l*(+)ornithine	11·3
	,, ,, 0·005 *M* *l*(+)citrulline	11·4
4	0·01 *M* NH_4Cl; 0·01 *M* pyruvate; 0·005 *M* *l*(+)ornithine	10·1
	,, ,, 0·005 *M* *l*(+)citrulline	10·1
5	0·01 *M* NH_4Cl; 0·01 *M* *l*(+)lactate; 0·003 *M* *l*(+)ornithine	15·1
	,, ,, 0·003 *M* *l*(+)citrulline	15·4

Urea formation in the presence of ketonic acids

Table 5 does not confirm Bach's statement [1939] that α-ketoglutarate and pyruvate increase the rate of urea formation from citrulline. I assume that this discrepancy is due to experimental errors in Bach's procedure. The total amount of urea-N in Bach's experiments was of the order of 100μg. (varying from 2 to 300μg.). This was

Table 5. *Effect of α-ketonic acids on urea formation in sliced rat liver*

Well-fed rats; incubation 90 min.; 40°.

Exp.	Substrates added	Q_{urea}
1	0·01 *M* *l*(−)leucine	0·40
	,, 0·005 *M* pyruvate	0·43
	,, 0·005 *M* oxaloacetate	0·49
	,, 0·005 *M* α-ketoglutarate	0·34
	0·01 *M* *l*(+)glutamate	0·41
	0·01 *M* *l*(−)aspartate	0·65
	0·01 *M* *l*(+)alanine	0·71
	0·01 *M* *l*(+)alanine; 0·005 *M* oxaloacetate	0·82
	0·01 *M* *l*(+)alanine; 0·005 *M* α-ketoglutarate	0·56
2	0·01 *M* *l*(+)citrulline	2·82
	,, 0·01 *M* pyruvate	2·58
	,, 0·01 *M* α-ketoglutarate	2·85
3	0·01 *M* *l*(+)citrulline	2·08
	,, 0·01 *M* pyruvate	1·60
	,, 0·01 *M* α-ketoglutarate	1·79

measured by titration with 0·01*N* HCl, 100μg. N being equivalent to 0·7 ml. 0·01*N* HCl. Only an aliquot of 30% was used for the urea determination so that the actual titration reading was on average no more than 0·2 ml. 0·01*N* HCl. It can hardly be expected that this procedure yields very accurate results. This is also borne out by Bach's own quadruplicate determinations: four lots of slices from the same liver (which ought to give identical results) gave 0, 10, 20 and 110μg. urea N [see Bach, 1939, p. 1835, Table I, Exp. X, 'before incubation' column]. The effects on which Bach's schemes are based are of the same order (44–95μg. urea N) and therefore fall within the limits of error. But it may also be pointed out that even were Bach's results real, they could hardly be regarded as evidence in support of his schemes.

Bach also claims to have observed 'a marked increase in urea formation from glutamine + NH_4Cl when Na formate was added'. He thought that Na formate might be a source of carbon in the urea synthesis. I find no effects of formate on the rate of urea synthesis under the conditions stated by Bach. In fact there appears to be so far no evidence indicating that formate is metabolized in mammalian tissues. Moreover, the experiments of Evans & Slotin [1940] and of Rittenberg & Waelsch [1940] with isotopic carbon finally prove that carbon atom of urea is derived from CO_2 and bicarbonate.

Trowell's experiments and interpretations

Trowell [1942] found with perfused rat liver: (1) that the rate of urea formation from arginine was sometimes lower than the rate of urea formation from NH_3 in the presence of ornithine; (2) that citrulline and NH_3 yielded urea (as predicted by the ornithine cycle) but that the effect of citrulline was 'transitory'; (3) that citrulline had no catalytic effect on urea formation.

I have no reason to doubt the correctness of Trowell's experimental observations, but I am unable to accept his conclusion that the theory of the ornithine cycle is 'untenable'. The theory does not postulate that under each and every condition arginine yields urea more rapidly than ornithine and NH_3, or that the citrulline effect is 'permanent', or that citrulline has a catalytic effect. Trowell chose most unfavourable conditions. Instead of using equimolecular quantities when comparing the rates of urea formation from arginine on one hand and from ornithine + NH_3 on the other, he used about ten times less arginine than NH_3.* Under these conditions the relatively low rate of urea formation from arginine is irrelevant. I find transitory effects of citrulline on urea formation when there is no excess of NH_3. The effect of citrulline then comes to a standstill, as is expected, when the NH_3 is exhausted and I presume that this occurred in Trowell's experiments. There

* Trowell has pointed out (Discussion at the meeting of the Biochemical Society at Manchester on July 10th, 1942) that the arginine concentration in his experiments was equivalent to the ornithine concentration in control experiments. Thus the arginine added was equivalent to the maximum amount of arginine that could have been formed from the added ornithine. This procedure does not ensure that the *concentrations* of arginine at the site of the urea synthesis are the same when ornithine and arginine are added. Arginine synthesized from ornithine in the liver would be concentrated at a specific site whilst added arginine is more or less evenly distributed over the whole perfusion system. The affinity of arginase for its substrate is remarkably low. At *p*H 9·2 a concentration of about 3% arginine is necessary to saturate the enzyme [Van Slyke, 1942]. The concentration of arginine will thus greatly influence the rate of its hydrolysis.

These considerations apply generally to experiments carried out on heterogeneous systems, such as perfused or sliced tissues, and it is therefore of no consequence if a metabolite has to be added in comparatively high concentrations to produce an effect. Trowell confirms that at certain concentrations arginine, or citrulline plus NH_3, yield urea as postulated by the theory. As the 'physiological' concentrations of the reactants are unknown I regard it as irrelevant what concentrations have to be added. It should be pointed out that those who do not agree with this, imply that the highly specific enzyme systems which at suitable concentrations produce urea from arginine, or from citrulline plus NH_3, are unrelated to the normal urea production, and that their parallel occurrence with urea synthesis is accidental.

The concentrations which produce effects will depend, among other factors, on the affinity of the substrate for the enzyme. If the critical concentrations are different for ornithine, citrulline and arginine (as Trowell's data indicate) this suggests different affinities of the substrates for their respective enzymes.

are no indications in his paper suggesting that enough NH_3 was present. As regards Trowell's failure to detect a catalytic effect of citrulline, it must be borne in mind that a catalytic effect of ornithine or citrulline is not expected in every experiment because the synthesis of urea is not the only metabolic process in liver in which ornithine and citrulline take part. There are other reactions, probably many, in which these amino acids are used up, e.g. the synthesis of protein [see Madden & Whipple, 1940], the oxidative degradation [see Krebs, 1933] and the conversion into other amino acids [see Krebs, 1939; Clutton, Schoenheimer & Rittenberg, 1940; Roloff, Ratner & Schoenheimer, 1940]. These reactions are responsible for the fact that ornithine and citrulline are never ideal catalysts (i.e. catalysts acting indefinitely). Even the complete absence of 'catalytic' effects under certain conditions is therefore no argument against the ornithine cycle, for it can be explained by side reactions which remove the catalyst. Although such side reactions probably occur in every liver, it is not difficult to demonstrate catalytic effects of ornithine and citrulline. Suitable conditions were described by Krebs & Henseleit [1932*c*]. These included the addition to the medium of lactate, which Trowell omitted. Presumably lactate suppresses one of the reactions destroying ornithine—the oxidative breakdown [see Krebs, 1935*a*]. If lactate is present catalytic effects with citrulline are usually found as shown in the example quoted in Table 6. Similar effects were found in six out of eight experiments.

Table 6. *Catalytic effect of citrulline on urea formation in rat liver*

3 ml. medium; 0·01 *M* lactate; 0·01 *M* NH_4Cl; incubation 120 min.

Further substrates (final concentrations)	mg. tissue	μl. urea formed	Urea formation caused by 7μl. citrulline or ornithine μl.	Q_{urea}
None	4·12	20·4	—	2·5
10^{-4} *M* citrulline	7·45	125	88	8·4
10^{-4} *M* ornithine	4·80	96	72	10·0

Some general remarks about the theory of urea formation

Has the ornithine cycle been 'proved'? Trowell [1942] writes of the ornithine cycle, and of other schemes, 'no one of them has been proved ever to occur'. Similar statements are not infrequently made and it may therefore not be superfluous to review the position.

The ornithine cycle is a theory; it is a supposition designed to account for a number of observations and to serve as a starting point for further investigations. It was first proposed [Krebs & Henseleit, 1932*a*] to explain two facts concerning the formation of urea in the liver: (1) the catalytic effect of ornithine, (2) the abundant occurrence of arginase in the liver of 'ureotelic' organisms. On the basis of the theory a third fact was soon discovered, viz., the ability of the liver to form urea from citrulline and NH_3 [Krebs & Henseleit, 1932*b*] and this made it possible to elaborate details of the theory. Three further relevant observations which were subsequently made on the basis of the theory are the following: the occurrence in liver of the reaction ornithine $+ NH_3 \rightarrow$ arginine was demonstrated with the help of isotopic hydrogen and nitrogen [Clutton *et al.* 1940; Schoenheimer & Rittenberg, 1940]; the occurrence in liver of the reaction ornithine $+ NH_3 \rightarrow$ citrulline was shown by Gornall & Hunter [1941]; the carbon atom of urea was shown to be derived from CO_2 [Evans & Slotin, 1940; Rittenberg & Walesch, 1940]. These observations 'prove', i.e. establish as a fact, the occurrence of each step of the cycle as postulated by the theory. This of course is true only for the conditions of the experiments and it can be said with some justification that the reactions have not been proved to occur under 'physiological' conditions, i.e. in the intact liver in situ. In all experiments some facts were 'unphysiological'. The liver was taken out of the body and sliced or artificially perfused. The concentrations of the reactants were artificially raised. Hence

the statement that the ornithine cycle is operative in the liver in situ implies the assumption (which some do not wish to make) that the cycle occurs under conditions different from those of the experiments. In this sense it may be formally correct to say that the cycle has not been proved to occur in 'normal' liver, but such a statement is of little scientific value for it applies to every intermediary reaction mechanism. It is impossible to investigate these mechanisms under 'physiological' conditions. Experiments can only prove that a tissue has the ability to perform certain chemical reactions, but if these reactions are intermediary stages, their 'normal' occurrence cannot be proved. In making the assumption that the experimental results can be applied to 'normal' conditions 'investigators have relied upon the fact that the body, though the seat of a myriad reactions and capable perhaps of learning, to a limited extent and under stress of circumstances, new chemical accomplishments, is in general able to deal only with what is customary to it' [Hopkins, 1913].

Alternative theories. If a new fact is brought to light which cannot be explained by, or even appears to be incompatible with, the ornithine cycle, one of the following three assumptions may be made:

(1) that the theory is untenable [see Trowell, 1942];

(2) that there are alternative mechanisms of urea synthesis [see Leuthardt, 1938*a*; Bach, 1939];

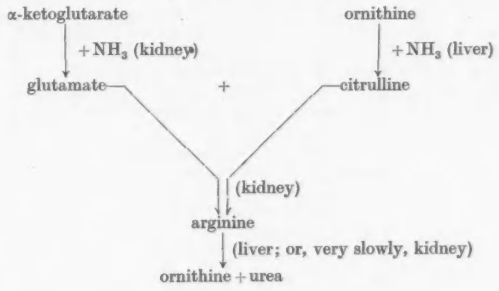
(3) that the theory is incomplete and has to be modified and extended.

Assumption (1) is not satisfactory as long as there is nothing to take the place of the ornithine cycle. A theory which explains some facts and can be put to the test of experiment is better than no theory at all. Assumption (2) is an *ad hoc* assumption and on general grounds ('Occam's Razor') such assumptions should not be made if they are unnecessary, i.e. if the observations are accounted for by one theory. Assumption (3) is the procedure of choice when the theory is already well established as is the ornithine cycle, and it should be explored to the full before the alternatives are considered. It has always been obvious that the theory of the ornithine cycle is incomplete; for example it makes no reference, in its present form, to the mechanism of transmission of energy, to the enzymes which activate ornithine, citrulline and possibly NH_3, and to the parallel reactions in which the substances of the ornithine cycle participate.

But if there is so far no case for alternative mechanisms of urea formation in the mammalian liver, it is already clear that in certain circumstances urea may be formed by a modification of the cycle. As mentioned earlier in this paper, arginine can arise from citrulline in two different ways:

Citrulline + NH_3 → arginine (liver),
Citrulline + glutamic acid → arginine (kidney).

Furthermore, kidney is known to form glutamic acid from ammonium α-ketoglutarate [Krebs & Cohen, 1939] and the combined action of liver and kidney can therefore yield urea in the following way:



In this modification the amino group of glutamic acid can yield urea without NH_3 acting as an intermediate, and some NH_3 can be converted into urea with glutamic acid acting as an intermediate. But it is certain that this modification is of no importance in the normal synthesis of urea. The significance of the synthesis of arginine from glutamic acid and citrulline, lies in the part which it plays in the synthesis of creatine [Borsook & Dubnoff, 1941].

SUMMARY

1. Leuthardt's observation that liver of starved guinea-pigs forms urea more rapidly from glutamine than from ammonium chloride is confirmed, but it is shown that the rate of urea formation from ammonium glutamate is of the same order as that from glutamine. The facts are in accordance with the view that ammonium glutamate is an intermediate in the urea formation from glutamine. It is therefore unnecessary to assume a more or less direct conversion of the amide N of glutamine into urea, as postulated by Leuthardt.

2. The ability of kidney cortex to synthesize arginine from citrulline and glutamate, discovered by Borsook & Dubnoff, is confirmed. The synthesis does not occur in the liver and therefore cannot be responsible for the accelerating effect of glutamate on the urea synthesis in this tissue.

3. Bach's statement that α-ketoglutarate and pyruvate increase the rate of urea formation from citrulline and from ornithine is not confirmed. The discrepancy is assumed to be due to experimental errors in Bach's procedure.

4. The results of Trowell, who claimed to have found that under certain conditions arginine and citrulline do not behave in the manner postulated by the ornithine cycle theory, are discussed, and reasons are given which show that Trowell's findings are not contradictory to the theory.

This work was aided by grants from the Rockefeller Foundation and from the Medical Research Council.

REFERENCES

Akasi, S. [1937]. *J. Biochem., Tokyo*, **26**, 129.
Bach, S. J. [1939]. *Biochem. J.* **33**, 1833.
Baldwin, W. [1935]. *Biochem. J.* **29**, 252.
Borsook, H. & Dubnoff, J. W. [1941]. *J. biol. Chem.* **141**, 717.
Calvery, H. O. & Block, W. D. [1934]. *J. biol. Chem.* **107**, 155.
Clutton, R. F., Schoenheimer, R. & Rittenberg, D. [1940]. *J. biol. Chem.* **132**, 227.
Damodaran, M. & Narayanan, K. G. A. [1940]. *Biochem. J.* **34**, 1449.
Edlbacher, S. & Bonem, P. [1925]. *Hoppe-Seyl. Z.* **145**, 69.
Edlbacher, S. & Burchard, H. [1931]. *Hoppe-Seyl. Z.* **194**, 69.
Edlbacher, S. & Zeller, A. [1936]. *Hoppe-Seyl. Z.* **242**, 253.
Edson, N. L. [1935]. *Biochem. J.* **29**, 2082.
Evans, E. A. jr. & Slotin, L. [1940]. *J. biol. Chem.* **136**, 805.
Felix, K. & Müller, H. [1936]. *Hoppe-Seyl. Z.* **240**, 1.
Felix, K., Müller, H. & Dirr, K. [1928]. *Hoppe-Seyl. Z.* **178**, 192.
Felix, K. & Schneider, H. [1938]. *Hoppe-Seyl. Z.* **255**, 132.
Gornall, A. G. & Hunter, A. [1941]. *Biochem. J.* **35**, 650.
Hellermann, L. & Stock, W. C. [1938]. *J. biol. Chem.* **125**, 771.
Hopkins, F. G. [1913]. *Rep. 83rd Meeting Brit. Ass. Adv. Science*, p. 652.
Hunter, A. [1938]. *Biochem. J.* **32**, 826.
Hunter, A. & Pettigrew, J. B. [1937]. *Enzymologia*, **1**, 341.
Hunter, A. & Woodward, H. E. [1941]. *Biochem. J.* **35**, 1298.
Krebs, H. A. [1933]. *Hoppe-Seyl. Z.* **217**, 191.
—— [1934]. *Enzymologia*, **3**, 247.
—— [1935*a*]. *Biochem. J.* **29**, 1620.
—— [1935*b*]. *Biochem. J.* **29**, 1951.
—— [1939]. *Enzymologia*, **7**, 53.

Krebs, H. A. & Cohen, P. P. [1939]. *Biochem. J.* **33**, 1895.
Krebs, H. A. & Henseleit, K. [1932*a*]. *Klin. Wschr.* **11**, 757.
—— —— [1932*b*]. *Klin. Wschr.* **11**, 1137.
—— —— [1932*c*]. *Hoppe-Seyl. Z.* **210**, 33.
Leuthardt, F. [1938*a*]. *Hoppe-Seyl. Z.* **252**, 238.
—— [1938*b*]. *Biochem. Z.* **299**, 280.
Madden, S. C. & Whipple, G. H. [1940]. *Physiol. Rev.* **20**, 195.
Rittenberg, D. & Waelsch, H. [1940]. *J. biol. Chem.* **136**, 799.
Roloff, M., Ratner, S. & Schoenheimer, R. [1940]. *J. biol. Chem.* **136**, 561.
Schoenheimer, R. & Rittenberg, D. [1940]. *Physiol. Rev.* **20**, 218.
Trowell, O. A. [1942]. *J. Physiol.* **100**, 432.
Van Slyke, D. D. [1942]. *Advances in Enzymology*, **2**, 33.
Weil, L. & Russell, M. A. [1934]. *J. biol. Chem.* **106**, 505.

Note added 22 October 1942. Bach & Williamson recently reported (meeting of the Biochemical Society at Oxford, 26 September 1942) that 1·6% ornithine inhibits arginase in rat liver slices but has no effect on the urea synthesis from ammonium lactate. They conclude that arginine is not an intermediate in the urea production from ammonium lactate.

An alternative interpretation is the following: the rate of urea formation from arginine in the experiment of Bach & Williamson was extremely low. Thus only a small fraction of the liver arginase can have been active, presumably only the arginase of the outer layers of the slice and of disintegrated cells. The bulk of the slice did not participate in the hydrolysis of arginine, or else the rate of urea formation from arginine would have been 20 to 100 times higher. This is probably due to the slow diffusion of arginine into the slice. The experiment therefore does not prove that 1·6% ornithine inhibits arginase 'in slices'.

I find that the urea synthesis from ammonium lactate is inhibited by 1·6% ornithine when slices are kept in a more alkaline medium (1% CO_2 instead of 5% CO_2) where ornithine (because of a decrease in its ionisation) is expected to permeate more readily into the cells. The argument of Bach & Williamson would lead to the conclusion that in the more alkaline medium arginase participates in the urea synthesis from ammonium lactate, or, in other words, that the mechanism of urea formation varies with the H ion and CO_2 concentrations.

87. The Effect of Oxidants and Reductants on some Sugar Hydrolysing Enzymes

By **Jacob Fiegenbaum**, *Chemical Department, Cancer Research Laboratories, The Hebrew University, Jerusalem*

(*Received 14 August 1942*)

Leibowitz [1925] and Leibowitz & Mechlinsky [1926] advanced the theory of a specific gluco-maltase in taka-diastase distinct from yeast maltase and from taka-sucrase [Hestrin, 1940]. According to Weidenhagen [1932; 1933] there is only one maltase identical with gluco-sucrase. Leibowitz & Hestrin [1938; 1939] found that taka-maltase and sucrase can be differentiated through the greater thermostability and acid stability of the former.

In order to differentiate further between the two hydrolysing capacities of taka-diastase, we studied the effect of oxidation and reduction on the enzymic preparation.

Experimental

In our experiments we used commercial samples of taka-diastase, Parke-Davis and Co.

As oxidant and reductant we used H_2O_2 and $Na_2S_2O_4$ respectively.

The enzyme solutions were prepared by dissolving 2·5 g. of the commercial product in 15 ml. distilled water in a cellophane bag. The solution under toluene was dialysed at room temperature for 1 day against running tap water and 1 day against distilled water, which was changed several times until the solution was free of reducing constituents. The solution was filtered and brought to 20 ml.

4·5 ml. samples of the enzyme solution were mixed in test-tubes, with 0·5, 1 and 1·5 ml. H_2O_2 30% (perhydrol Merck 'speciale pour le Tropique') respectively; all the samples, including a control solution without H_2O_2, were brought to 6 ml. with distilled water. After 24 hr. at room temperature, the H_2O_2 was removed by dialysing all solutions in cellophane bags for 2 days against distilled water, which was changed several times. The samples again were brought to the same volume.

The same procedure was used for preparing an enzyme solution treated with hydrosulphite ($Na_2S_2O_4$). A 3% $Na_2S_2O_4$ of Schering-Kahlbaum was used.

As *substrates* we used 4% solutions of maltose and sucrose (Schering-Kahlbaum) dissolved in Sørensen's phosphate buffer of *p*H 4·5 for maltose and *p*H 5·5 for sucrase.

The enzyme mixture was made by mixing 0·5 ml. of the enzyme solution with 4·5 ml. substrate solution in glass stoppered test tubes. The incubation temperature was 37°.

The hydrolysis of the sugars was determined according to Lehmann-Maquenne-Schoorl in 1 ml. portions of enzyme mixture removed after different time intervals and introduced into 1 ml. 2% Na_2CO_3 solution (to stop the enzyme activity).

Effect of H_2O_2 on the rate of maltose and sucrose hydrolysis. As shown in Figs. 1 and 2, taka-maltase is far less affected than sucrase. The inhibition of taka-sucrase is not substantially increased with increasing concentration of H_2O_2.

Effect of $Na_2S_2O_4$ on the rate of maltose and sucrose hydrolysis. Figs. 3 and 4 show that whereas taka-maltase is slightly stimulated by $Na_2S_2O_4$, taka-sucrase is inactivated, the inactivation increasing with the quantity of $Na_2S_2O_4$.

In order to determine whether the effects of H_2O_2 and $Na_2S_2O_4$ respectively are reversible, each once-treated solution was subjected to treatment with the reactant not previously used. Thus taka-diastase treated with H_2O_2 was subsequently treated with

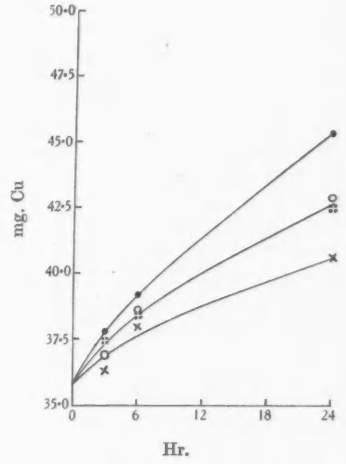


Fig. 1. Taka-maltase. • control; ○ 0·5 ml. H_2O_2 30%; ▪ 1 ml. H_2O_2 30%; × 1·5 ml. H_2O_2 30%.

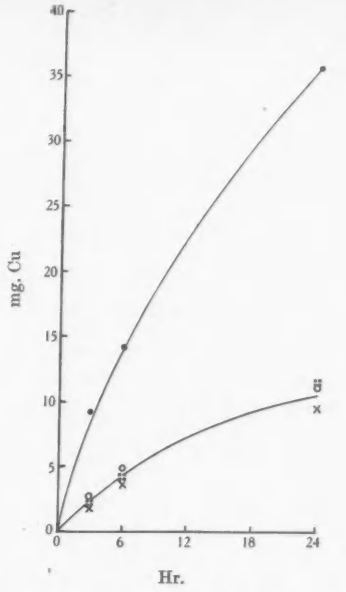


Fig. 2. Taka-sucrase. • control; ○ 0·5 ml. H_2O_2 30%; ▪ 1 ml. H_2O_2 30%; × 1·5 ml. H_2O_2 30%.

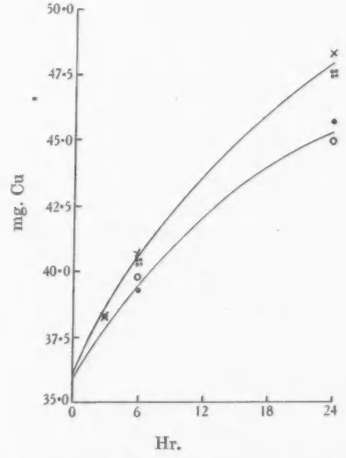


Fig. 3. Taka-maltase. • control; ○ 0·5 ml. $Na_2S_2O_4$ 3%; ▪ 1 ml. $Na_2S_2O_4$ 3%; × 1·5 ml. $Na_2S_2O_4$ 3%.

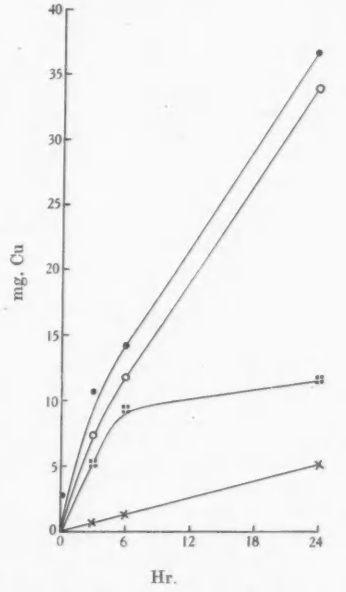


Fig. 4. Taka-sucrase. • control; ○ 0·5 ml. $Na_2S_2O_4$ 3%; ▪ 1 ml. $Na_2S_2O_4$ 3%; × 1·5 ml. $Na_2S_2O_4$ 3%.

$Na_2S_2O_4$, and vice versa. 5 ml. of the original dialysed solution of taka-diastase was treated for 24 hr. with 1·5 ml. 3% $Na_2S_2O_4$ dialysed until free of hydrosulphite, treated with 1·5 ml. H_2O_2 30% for 24 hr. again dialysed and brought up to the same volume with distilled water. In a second experiment the order of treatment was reversed.

Table 1. *Taka-maltase*

Hours	mg. Cu		
	I	II	III
0	31·6	31·6	31·6
3	32·2	32·8	31·6
6	32·2	33.7	31·6
24	36·2	37·5	33·7

Table 2. *Taka-sucrase*

Hours	mg. Cu		
	I	II	III
0	0·00	0·00	0·00
3	1·57	0·00	0·00
6	4·60	0·00	0·00
24	19·55	0·00	0·00

I, Control. II, $Na_2S_2O_4$ 3% and subsequently H_2O_2 30%. III, H_2O_2 30%, $Na_2S_2O_4$ 3%.

The results (Tables 1 and 2) show a striking difference in the behaviour of the two enzymes: in the case of maltase only the effect of the reagent used first was manifested. It follows that treatment of the maltase, whether by H_2O_2 or $Na_2S_2O_4$ renders the enzyme insensible to the effect of the other reagent. In the case of sucrase, an accumulation of inhibiting effects of both reagents was observed, so that by treatment of taka-diastase with H_2O_2 and $Na_2S_2O_4$ or by $Na_2S_2O_4$ and H_2O_2 in sequence a complete inactivation of the sucrase was attained, whereas the maltase still retained its activity.

In order to ascertain how sucrase from a different source is affected by the oxidising and reducing agents, we used a 1% yeast autolysate (for which I am indebted to Dr J. Leibowitz) containing practically no maltase.

5 ml. samples of the yeast extract were mixed in different test-tubes, one with 1 ml. H_2O as control solution, one with 1 ml. 3% $Na_2S_2O_4$, and a third with 1 ml. 30% H_2O_2. After 24 hr. at room temperature, the samples were dialysed until no $Na_2S_2O_4$ or H_2O_2 was detected.

Table 3. *Yeast sucrase*

Hours	mg. Cu		
	H_2O	$Na_2S_2O_4$	H_2O_2
0	0·00	0·00	0·00
3	4·46	12·80	0·00
6	7·74	23·20	0·00
24	37·00	60·00	0·00

Table 3 shows that yeast sucrase is completely inhibited by H_2O_2 but is strikingly activated by $Na_2S_2O_4$.

Discussion

Taka-maltase, taka-sucrase and yeast sucrase were investigated as to the effect on them of treatment with H_2O_2 (as an oxidant) and $Na_2S_2O_4$ (as reductant), used separately or in succession. Each enzyme showed its own peculiar specific behaviour. Taka-maltase was markedly inhibited by H_2O_2 and slightly stimulated by $Na_2S_2O_4$. Taka-sucrase was strongly inhibited and sometimes completely inactivated by both reagents. Yeast sucrase was strongly stimulated by $Na_2S_2O_4$ and completely destroyed by H_2O_2.

It is always a question whether a different response of two enzymes to the same physical or chemical agent is due to differences in the nature of the active principle of the enzyme molecule or of the accompanying substances ('carriers') which in most cases constitute the bulk of the substance in the active preparation. This uncertainty applies to a comparison of sucrase from *Aspergillus oryzae* and that from yeast. Since, however, taka-sucrase and taka-maltase belong to the same preparation, it follows clearly that the differences detected between them are due to the nature of these enzymes themselves.

The different behaviour of the taka-enzymes towards oxidizing and reducing agents was accentuated in successive treatment with $Na_2S_2O_4$ and H_2O_2. In the case of sucrase, the second treatment simply added inhibiting effect to the inhibition already caused. The behaviour of maltase was quite different and very peculiar: inhibition through peroxide was not evoked or compensated by subsequent treatment with hydrosulphite, though the latter itself has a stimulating effect on the enzyme. If the enzyme was treated previously with $Na_2S_2O_4$, the stimulation caused was not diminished by the subsequent treatment with the inhibitor H_2O_2. It appears that treatment by each of the two agents so affects the enzyme molecule as to render it insensitive to the effect of the other. It is difficult at the moment to give an explanation for this strange phenomenon.

Taken together, the facts obtained by the treatment of taka-diastase by oxidants and reductants, confirm the theory that the maltose and sucrose splitting capacities of taka-diastase must be attributed to two distinct and specific disaccharases.

Summary

1. Taka-maltase is markedly inhibited by H_2O_2 and slightly stimulated by $Na_2S_2O_4$.

2. Taka-sucrase is strongly inactivated by both the oxidizing and reducing agents.

3. Yeast sucrase is strongly activated by hydrosulphite and completely inactivated by hydrogen peroxide.

4. In the event of successive treatment by both reagents in any order the inhibiting effects are cumulative in the case of taka-sucrase, while in taka-maltase only the effect of the initial treatment (whether stimulation by $Na_2S_2O_4$ or inhibition by H_2O_2) is manifested.

5. Support is lent to the theory that taka-maltase and taka-sucrase are two distinct and specific disaccharases.

REFERENCES

Hestrin, S. [1940]. *Enzymologia*, **6**, 193.
Leibowitz, J. [1925]. *Hoppe-Seyl. Z.* **149**, 184.
Leibowitz, J. & Mechlinsky, P. [1926]. *Hoppe-Seyl. Z.* **154**, 64.
Leibowitz, J. & Hestrin, Sh. [1938]. *Nature, Lond.*, **141**, 552.
—— —— [1939]. *Nature, Lond.*, **143**, 339.
Weidenhagen, R. [1932]. *Ergebn. Enzymforsch.* **1**, 169.
—— [1933]. *Hoppe-Seyl. Z.* **216**, 255.

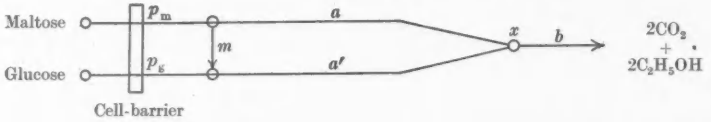
88. The Direct Fermentation of Maltose by Yeast. 2*

By **J. Leibowitz** and **S. Hestrin**, *Department of Hygiene and Bacteriology, and Chemistry Department, Cancer Laboratories, The Hebrew University, Jerusalem*

(*Received 20 August 1942*)

Discussion on the existence of disaccharide-specific zymase systems appears to be entering a new phase. The fermentative breakdown of maltose, sucrose, lactose and trehalose has been investigated in recent years from different directions. Nevertheless, the question whether hydrolysis is an indispensable preliminary to the fermentation of a disaccharide is still variously answered. Supporters of the view that disaccharide fermentation may be 'direct' have as a rule been content, hitherto, with negative proofs showing that oligases are not indispensable to disaccharide fermentation. There is clearly need, however, for a positive hypothesis concerning the mechanism whereby disaccharides could be fermented directly.

Hydrolysis to glucose appears to be one way but not the only way in which maltose is broken down by the yeast cell. Pictorially this view may be represented as follows:



Here p_m and p_g are the respective permeation processes whereby maltose and glucose become available to the action of intracellular enzymes. m is the hydrolytic reaction mediated by maltase and thus the path followed in indirect maltose fermentation. a' and b represent the reaction sequence of glucolysis; a and b represent the reaction sequence of direct maltose fermentation to be referred to below as maltolysis. The unidentified intermediary in which the direct fermentation paths of maltose and glucose presumably merge has been designated as x. Following Willstätter, the enzyme systems responsible for *direct* fermentation of maltose and glucose are termed maltozymase and glucozymase respectively.

Let us term the over-all velocity of maltose fermentation and glucolysis respectively V_m and V_g. Conceivably the ratio V_m/V_g may be equal to, greater than, or less than unity. In considering the bearing of kinetic evidence on the possible existence or non-existence of a direct maltose fermentation, careful distinction between these three cases will be useful.

(1) $V_m/V_g<1$. Since in this case maltose is fermented more slowly than glucose, the rate-limiting part-reaction of the maltose fermentation cannot be contained in the glucolytic sequence *a' and b*. If a given activator or inhibitor of maltose fermentation, but not of glucose fermentation, is found to be without action at stages p_m and m of the scheme presented, the existence of this specific activator or inhibitor must then be regarded as convincing, if indirect, evidence of the existence in the cell of a specific sequence a of maltolysis.

(2) $V_m/V_g=1$. Unless evidence is advanced to the contrary, it is plausible to assume in this case that the rate-limiting reaction of maltose and glucose fermentation is the same, i.e. that the rate-limiting reaction is contained in the common component b. As

* Cf. preliminary note [Leibowitz & Hestrin, 1940], earlier investigation [Leibowitz & Hestrin, 1939], and dissertation by Hestrin [1941].

long as *b* continues to set the pace, the presence of specific activators or inhibitors of maltolysis will not affect the rate of gas liberation.

(3) $V_m/V_g > 1$. The simplest explanation of this case is to assume that *a* exists and that its velocity is greater than *a'*, the latter being rate limiting for glucolysis but not for maltose fermentation. This interpretation is only necessary, however, under certain conditions. Their specification will not be necessary in the present connexion. As in case (2) the limiting part-reaction of maltose fermentation may be contained in *b*. While the latter remains limiting, specific activators and inhibitors of maltose fermentation need not necessarily affect the rate of gas liberation.

Conditions as specified under (1) were found in baker's yeast; conditions as specified under (2) and (3) in a brewer's yeast.

Methods

Yeast. Except where otherwise stated, the yeast used for tests was a Palestinian brand of commercial baker's yeast supplied by Paca Ltd. Samples purchased at different dates were left to stand on ice for a number of days before use. This treatment lessened the fermentative power of the yeast to maltose, augmented the effect on maltose fermentation of exposure of the yeast to glucose, and enhanced certain inhibitory effects described in the sequel. Autofermentation was always found to be negligible. Samples of brewer's yeast, Rishon strain, were kindly provided by Dr Hayman of Palestine Brewery Ltd., Rishon.

Sugars. All sugars used were of good quality and supplied by B.D.H. or Schering-Kahlbaum.

Gasometers. Except where otherwise stated, fermentation was followed gasometrically using the apparatus described by Hestrin [1941]. Duplicate fermentation runs with this apparatus yielded results which were identical to within 3%. Aqueous 30% $CaCl_2$ served as the gasometer fluid.

Procedure. Mixtures were prepared for fermentation runs in 10 ml. capacity long-necked flasks and maintained in a water thermostat at 32°. 3 min. were allowed for temperature equilibration before taking zero reading. The flasks were shaken mechanically at a frequency of 70 oscillations per min. Dispersion of the yeast was further facilitated by the inclusion of several glass beads in the fermentation mixture.

Replication of experiments. All experiments reported are typical and representative of several which yielded an essentially identical result.

Units of measurement: 'gassing rate'. The measurements, being largely of relative rather than absolute importance, are presented uncorrected to standard conditions. To facilitate comparison between different experiments the gassing velocity has been expressed occasionally in terms of the maximum rate of gas evolution per unit fresh weight of yeast. Under suitable conditions this maximum is also the 'stationary' gassing rate.

Results

A. *Experiments with baker's yeast* ($V_m/V_g < 1$)

(*a*) *Fermentation rate at different initial concentrations of substrate.* The influence of substrate concentration on the fermentation process has been dealt with by a number of investigators, but their work has been largely restricted to glucose fermentation. Hopkins & Roberts [1935] found in corroboration of Slator [1908] that the gassing rate in glucose fermentation is independent of the initial substrate concentration within a wide range [cf. however, Nord & Weichherz, 1929].

Our baker's yeast, both fresh and after storage, behaved towards glucose in accordance with the findings of Hopkins & Roberts [1935]. The behaviour to maltose, on the other hand, was variable: fresh yeast cells generally fermented maltose at the same uniform

rate over a wide range of substrate concentrations. The stationary fermentation rate was always markedly below that obtained with glucose, strikingly variable with storage, and only set in after a relatively prolonged induction period. Using cell material which had been 'impoverished' by storage a definite diminution of the stationary gassing rate with increase in the initial concentration of maltose was frequently observed. Table 1 shows this retardant effect clearly. This retardation only becomes evident, it may be noted, after an initial delay.

Table 1. *Retardant effect of maltose on maltolysis by baker's yeast*

Solutions of 4·0 ml. containing 1 ml. McIlvaine's phosphate-citrate buffer *p*H 5·0, and sugar in the concentrations shown.

Initial conc. of maltose %	Total yield of CO_2 in ml.* after min.							Stationary† gassing rate ml./30 min./g. yeast
	75	165	195	225	255	285	315	
4·0	1·0	5·6	8·1	10·9 !	13·9	17·0	20·0	7·5
8·0	1·2	4·7	6·7 !	8·8	10·9	13·2	15·4	5·5
12·0	1·5	4·6 !	6·1	7·6	9·2	10·8	12·4	4·0
16·0	1·8	4·6 !	5·7	6·9	8·2	9·5	10·7	3·2

* ! indicates time of attainment of stationary gassing rate.

† Concentrations of glucose in the range of 2–16% were fermented at the uniform rate of 27·0 ml./30 min./g. yeast.

The measure of retardation of gassing rate by maltose is dependent on the physiological state of the cells. Marked retardation was frequent with cells which had been impoverished by storage. On the other hand, yeast which has been restored to full maltose fermenting capacity by treatment with glucose is not retarded or only slightly by high maltose concentration (Table 3 and Fig. 2). In view of this marked variability predictions as to the effect of concentration on maltose fermentation with types of yeast other than that used by us are hardly justified.

Mixtures of maltose and glucose were found to ferment at the same rate as glucose itself as long as an adequate concentration of glucose was maintained in the mixture. Maltose therefore did not act as an inhibitor in glucose fermentation.

The intimate mechanism of the maltose-concentration effect is obscure. This is not, however, the only known case of retardation of fermentation by a sugar. Slator [1908] found mannose a retardant of glucose fermentation, and glucose a retardant of galactose fermentation. Sobotka, Holzman & Reiner [1936] have described a retardation of hexose fermentation by pentoses. Experiments elsewhere show that under certain conditions glucose, but not maltose, may exert a retardant effect on fermentation by brewer's yeast [Hestrin, 1941].

(*b*) *Inhibition by methyl α-glucoside.* A substance analogous in certain features of structure to maltose is methyl α-glucoside. This has long been known to be an efficient retardant of invertase action [Michaelis & Pechstein, 1914] and has been recently found also to inhibit the oxidation of glucose by luminescent bacteria [Johnson & Anderson, 1938]. It seemed of interest therefore to examine its possible inhibitory action on sugar fermentations.

Under ordinary conditions the glucoside regularly failed to inhibit glucose fermentation by baker's yeast to a marked extent. Also sucrose fermentation was not noticeably affected by its presence. Raffinose fermentation, again, was slightly inhibited (experiments to be published elsewhere). Maltose fermentation, on the other hand, was strongly inhibited, both as to induction time and stationary gassing rate. Experiments showing the action of the glucoside under different conditions are summarized in Tables 2 and 3 and Fig. 1. The glucoside itself was not fermented at an appreciable rate under the conditions of these tests. Methyl β-glucoside, again, failed to inhibit maltose fermentation.

Table 2. *Substrate specificity, and influence of pH on inhibitory action on fermentation of methyl α-glucoside*

Suspensions of 8% yeast in mixture volumes of 4 ml. containing water or Sørensen phosphate buffer solution and 8% maltose or glucose with or without addition of glucoside. Temp. 22°. Van-Iterson-Kluyver gasometer. Intermittent shaking.

Substrate	Buffer	Methyl α-glucoside %	Total yield of CO_2 in ml. after hr. 1	3	8	24
Maltose	*p*H 4·5	0·0	0·2	1·7	9·2	23·2
	*p*H 4·5	4·0	0·1	0·7	4·8	11·1
Glucose	*p*H 4·5	0·0	5·9	16·2	—	—
	*p*H 4·5	4·0	4·1	14·4	—	—
Maltose	*p*H 7·0	0·0	0·0	0·3	2·6	11·5
	*p*H 7·0	4·0	0·0	0·0	0·2	2·4
Glucose	*p*H 7·0	0·0	5·0	14·0	—	—
	*p*H 7·0	4·0	4·8	14·3	—	—
Maltose	Water	0·0	0·1	0·5	7·2	23·0
	Water	4·0	0·0	0·0	3·9	11·4

Table 3. *Inhibition of maltose fermentation by methyl α-glucoside at different concentrations of maltose*

Mixture volume of 5·0 ml. containing 4 ml. KH_2PO_4, suitable concentrations of sugar, and 8% yeast. Yeast activated before experiment by allowing to stand in 0·9% glucose for 30 min.

Maltose %	Methyl α-glucoside %	Total yield of CO_2 in ml. after min. 30	60	90	120	150	180	Maximum gassing rate ml./30 min./g. yeast
5·0	0·0	1·8	3·8	8·2	14·2	21·6	29·4	19·5
5·0	4·0	1·0	2·7	5·4	8·7	13·0	18·2	13·0
7·5	0·0	2·1	5·6	10·5	16·7	24·0	31·8	19·5
7·5	4·0	1·2	3·7	7·5	12·3	17·6	23·6	15·0
10·0	0·0	2·3	6·2	11·5	17·6	24·9	32·5	19·0
10·0	4·0	1·9	5·0	9·0	13·6	19·2	25·3	15·2

It may be seen from the experiments reported that the action of the glucoside is a function of its concentration (Fig. 1) and also, though to a less marked degree, a function at a given concentration of glucoside of the latter's ratio to maltose (Table 3). The effect of *p*H on the inhibition is clearly shown in Table 2. In neutral phosphate solution, maltose fermentation itself is greatly depressed, but the relative effect of the glucoside is enhanced.

Since the possibility was remotely present that the inhibition was due to liberation of traces of methanol, experiments were undertaken to elucidate the effect of this alcohol on maltose fermentation. They showed that methanol in concentrations equivalent to 2–8% of glucoside does not inhibit but actually slightly favours maltose fermentation.

Attention may be drawn to the reversible character of the glucoside effect. If yeast cells which have been allowed to stand in a solution of the glucoside are separated out by centrifugation and washed they ferment pure maltose at their normal rate.

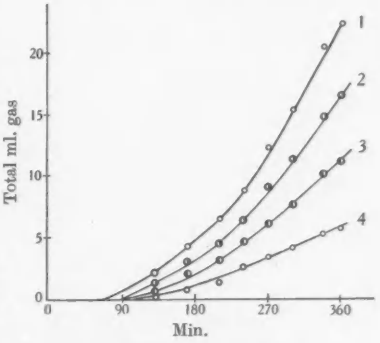


Fig. 1. Inhibition of maltose fermentation by different concentrations methyl α-glucoside. As in Table 4 but using storage-impoverished yeast. Initial concentration of maltose = 8%. Curve (1) 0·0%, (2) 2%, (3) 4%, (4) 8% glucoside.

As with the maltose inhibitory effect, that of the glucoside also is markedly dependent on the physiological state of the cells. Activation of the cells by addition of glucose

Table 4. *Effect of activation by glucose on inhibition of maltose fermentation by methyl α-glucoside*

4 ml. of mixture containing 2 ml. Sørensen phosphate *p*H 6·6, 10% yeast, and suitable concentrations of sugar. At this *p*H the glucose effect is pronounced.

Maltose %	Glucose %	Methyl α-glucoside %	Total yield of CO_2 in ml. after min. 30	60	95	110	125	170	200
8·0	0·0	0·0	0·0	1·2	3·0	4·4	6·1	12·6	17·6
8·0	0·0	4·0	0·0	0·0	0·0	0·0	0·0	0·8	1·2
8·0	0·5	0·0	2·7	4·7	8·8	10·6	12·8	20·3	25·5
8·0	0·5	4·0	3·0	4·7	6·9	8·0	9·4	13·7	17·2
0·0	0·5	0·0	2·2	2·3	—	—	2·4	—	2·4

Table 5. *Effect of previous fermentation on inhibition of maltolysis by methyl α-glucoside*

5 ml. of solutions containing 3 ml. Sørensen primary phosphate solution, 10% yeast, 8% maltose and the glucoside as indicated.

Time of glucoside addition (4%)*	Total yield of CO_2 after min. 60	90	120	150	180	200	% diminution of gas yield in 80 min. period following addition of glucoside
None	↓ 3·2	8·3	16·5	27·2	41·3	50·9	—
0 min.	↓ 0·2	1·1	4·3	10·8	18·8	26·3	88
120 min.	3·2	8·4	↓ 16·3	25·6	36·7	45·5	15

* ↓ denotes addition of glucoside.

markedly diminishes their susceptibility to inhibition by glucoside (Table 4). If the yeast cell is exposed to glucoside after maltose fermentation has set in, the inhibitory effect of the glucoside is almost negligible (Table 5).

(*c*) *The activation of maltolysis by zymohexoses.* The addition to maltose media of low concentrations of glucose, or of a factor such as maltase which produces glucose, has been shown to exert a remarkable stimulative influence on maltose fermentation, curtailing the induction time preceding the beginning of evolution of gas from maltose, and increasing the stationary gassing rate finally attained [Leibowitz & Hestrin, 1939; Schultz & Atkin, 1939; cf. also Blish & Sandstedt, 1937, and earlier contribution by Beijerinck, 1895].

A resolution of the dual effect of glucose addition has been achieved by variation of the initial maltose concentration. An experiment illustrating this point is summarized in Fig. 2. Glucose addition is seen to hasten the onset of maltose fermentation both at the higher and lower maltose concentration tested, but caused an appreciable increase in the ultimate gassing rate only when the higher maltose concentration inhibited fermentation.

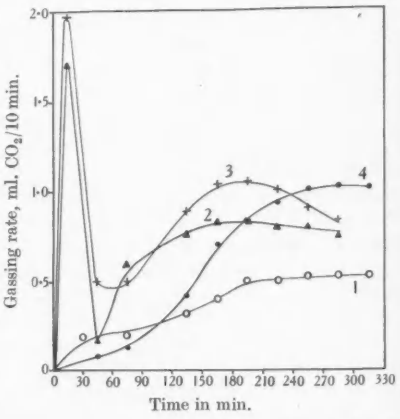


Fig. 2. Activation of maltolysis by glucose at different maltose concentrations. Composition as to buffer and yeast as in Table 1. Curve (1) 12% maltose; (2) 12% maltose and 0·5% glucose; (3) 4% maltose and 0·5% glucose; (4) 4% maltose.

The initial hump in the rate-time curves (2) and (3) of Fig. 2 is due of course to the greater fermentation rate of the rapidly disappearing glucose component. The drop in gassing rate found towards the end of the fermentation-run in the case of curve (3) is due to the approaching exhaustion of fermentable substrate.

It has been shown in the previous investigation, and it is here confirmed, that the effect of glucose is not bound up with the presence of the latter as such but persists in the yeast cell even when glucose has been removed from the medium. It seemed likely therefore that glucose acted by an influence exerted on the cell through glucolysis. Accordingly it seemed of interest to examine the effects on maltolysis of treatment of the yeast with other fermentable sugars and to compare their action with that of a normally non-fermentable sugar such as galactose. An experiment of this kind is presented in Table 6. A glance at the table shows that maltose fermentation is stimulated by fructose and mannose as well as by glucose but is not affected under the conditions of this experiment by galactose.

Table 6. *Activation of maltose fermentation by different sugars*

4·5 ml. solution containing 2·5 ml. Sørensen phosphate *p*H 6·5, 12% yeast, and suitable concentration of sugars.

Maltose %	Hexose 0·5%	Total yield of CO_2 in ml. after min. 60	120	180
6·0	None	0·0	0·1	1·0
6·0	Glucose	5·6	16·1	25·0
6·0	Fructose	4·7	11·0	20·7
6·0	Mannose	4·0	8·1	16·8
6·0	Galactose	0·0	0·0	0·8
0·0	Zymohexose*	2·0	2·0	2·0
0·0	Galactose	0·0	0·0	0·0

* Zymohexose = glucose, fructose or mannose.

(*d*) p*H relations*. The favourable effect of moderately acid *p*H on maltose fermentation was discovered by Willstätter & Bamann [1926] and has since been confirmed by different investigators [Sobotka & Holzman, 1934; Genevois, 1937; Schultz & Atkin, 1939]. The baker's yeast used in the present investigation showed the favourable effect of acid *p*H very clearly (e.g. in Table 3).

Experiments carried out towards the conclusion of this investigation revealed that the true nature of the *p*H-activity curve is obscured in the neutral range when a comparatively weak buffer is used. Thus, in neutral Sørensen phosphate, maltose fermentation is delayed but still possible. It could be shown, however, that the *p*H of these mixtures suffers a shift to the acid side as fermentation of maltose sets in. Inclusion of a little glucose in the fermentation mixtures hastens the onset of the acid shift [cf. Mirski & Wertheimer, 1939].

Using McIlvaine citrate-phosphate buffer, the acid shift from neutral was found to be largely eliminated. In this buffer precise determination of the variation of stationary gassing rate with *p*H in a sufficiently wide range of *p*H values becomes possible (*p*H 3·0–7·0). Typical experiments showing the dependence of fermentation on *p*H in adequately buffered medium are summarized in Fig. 3 (curves I and II).

It is apparent that whereas glucolysis by living baker's yeast is independent of the *p*H within the tested range of 3·0–7·0, maltolysis is optimal at *p*H 5·0, still considerable at *p*H 3·0, but completely suppressed under the conditions of the test at *p*H 7·0. Methyl α-glucoside was not fermented by fresh baker's yeast at any *p*H whatsoever under the conditions of the experiment summarized in Fig. 3.

Promaltose. It is important to note, particularly, in view of certain analytical methods of differentiating between maltose and glucose, that the negative fermentation reaction of baker's yeast to maltose and methyl α-glucoside at neutral *p*H is extremely

labile. Fresh, stored, and glucose-activated cells of baker's yeast failed to ferment maltose and the glucoside at neutral *p*H, but slight ability to ferment maltose at neutral *p*H was acquired by the yeast on prolonged incubation with maltose (over 8 hr.). Ability to ferment both maltose and glucoside at neutral *p*H was also conferred on the fresh yeast by drying (Table 7).

Table 7. *Fermentation of maltose and methyl α-glucoside by fresh and dried baker's yeast at neutral reaction*

Solution, 4·0 ml. containing 2·0 ml. McIlvaine phosphate-citrate buffer *p*H 7·0, 5% sugar, and 12% of fresh or air-dried baker's yeast.

Yeast	Substrate	Total yield of CO_2 in ml. after min. 20	40	60	95	115
Fresh	Maltose	0·0	—	0·0	—	0·0
Fresh	Methyl α-glucoside	0·0	—	0·0	—	0·0
Dried	Maltose	6·1	14·4	21·6	—	39·5
Dried	Methyl α-glucoside	1·2	2·4	3·6	6·4	8·2

Moreover, when fresh baker's yeast was analysed for maltase content by autolysis with ethyl acetate, as described by Willstätter & Steibelt [1921], a slight amount of

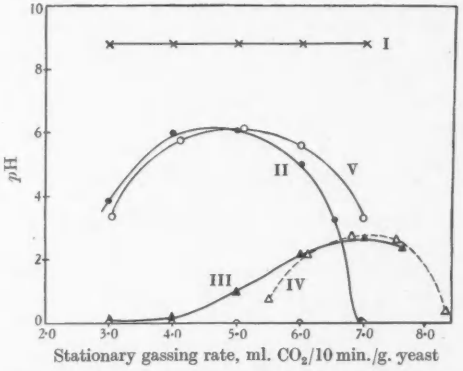


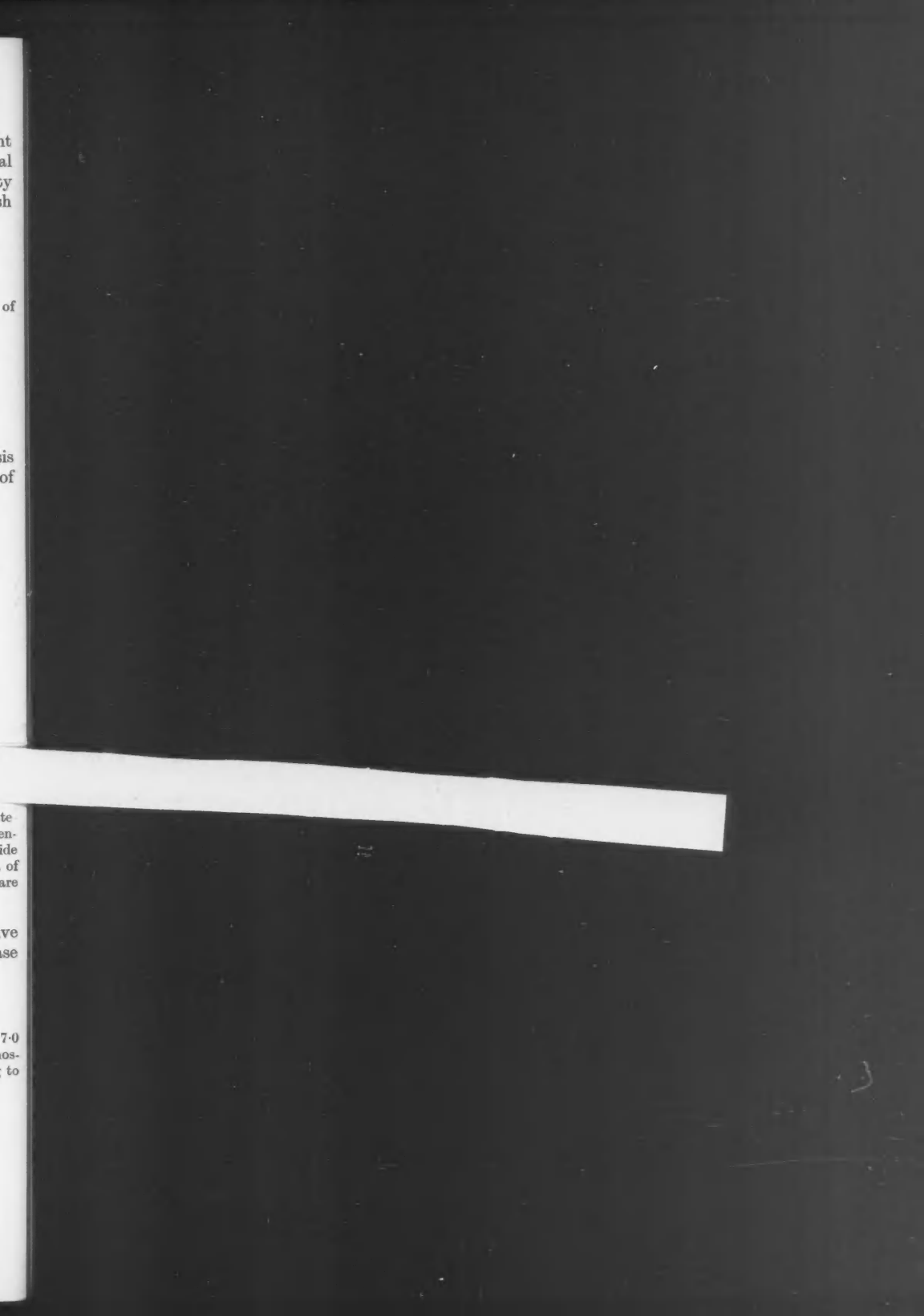
Fig. 3. Fermentation of sugars at different *p*H values. Solution, 5 ml. containing 2 ml. of McIlvaine citrate phosphate buffer, 6% yeast, and 6% of sugar substrate. Temperature 32°. Curve I, glucose fermentation by baker's yeast. Curve II, maltose fermentation by baker's yeast. Curve III, methyl α-glucoside fermentation by brewer's yeast. Curve IV, relative activity-*p*H curve of yeast maltase [from data of Willstätter & Bamann, 1926]. Curve V, maltose fermentation by brewer's yeast. Values plotted are relative. For absolute figures of gassing rate multiply by 31.

maltase was found to be liberated even though the fresh sample had given a negative reaction for maltose fermentation at neutral *p*H. The order of activity of this maltase solution to maltose, and the glucoside may be seen in Table 8.

Table 8. *Maltase of baker's yeast autolysate*

Autolysate prepared after Willstätter & Steibelt [1921] from baker's yeast negative to maltose at *p*H 7·0 in fermentation test. Solutions of 5·0 ml. total vol., containing 1·0 ml. autolysate, 2·0 ml. Sørensen phosphate buffer *p*H 7·0, and sugar as indicated. Incubation at 37°. Reducing power determined according to Bertrand on 2·0 ml. samples.

Substrate	Conc. in %	% hydrolysis after 24 hr.
Maltose	4	32
	8	16
Methyl α-glucoside	4	21



p. 778, Fig. 3. The figures on the abscissa should be interchanged with those on the ordinate.

A hypothesis which would explain these apparently paradoxical findings is that fresh baker's yeast contains maltase in the form of a zymogen which is converted into active maltase (=α-glucosidase) by treatments such as drying or autolysis. It is not unlikely that a similar explanation will apply to analogous findings with other oligase enzymes, e.g. the presence of lactase in preparations of *E. coli mutabile* variants which were inert to lactose [Deere, Dulaney & Michelson, 1939], trehalase in yeast which did not ferment trehalose [Myrbäck & Örtenblad, 1936], sucrase in autolysates of *Schizosaccharomyces octosporus* which does not ferment sucrose [Hofmann, 1934], cellobiase in preparations of lactose yeast which had failed to ferment cellobiose [Neuberg & Hofman, 1932], maltase in yeast which failed under anaerobic conditions to ferment maltose [Kluyver & Custers, 1940].

B. *Experiments with brewer's yeast* ($V_m/V_g \gtrless 1$)

(*a*) $V_m/V_g = 1$. Brewer's yeast, in media of the type employed in the foregoing, generally ferments maltose and glucose at the same uniform rate and after roughly equal, very brief inductions. With 9 out of 10 lots of yeast tested under these conditions the V_m/V_g ratio approximated closely to unity, and was practically independent of the concentrations of substrate employed for the comparison (2–12 %).

As was to be expected from considerations set forth in the introduction, maltose fermentation by this type of yeast was insusceptible of inhibition or activation by specific activators and inhibitors of maltolysis. Methyl α-glucoside or maltose in high concentration did not inhibit, and hexose addition did not activate, maltose fermentation by Richon brewer's yeast.

(*b*) $V_m/V_g > 1$. An exceptional lot of Richon yeast actually fermented maltose more rapidly than glucose. Experiments showing the fermentation of maltose and glucose by this yeast lot at different initial substrate concentrations and before and after ageing of the yeast are summarized in Fig. 4. It will be noted that ageing enhanced the relative superiority of the fermentation rate of maltose. It is also apparent that the ratio V_m/V_g is dependent on the initial substrate concentration at which the fermentation rates are determined. In contrast to ordinary brewer's yeast lots which fermented glucose as well as maltose at a uniform rate in a range of 2–12 %, the unique sample in question fermented glucose less readily at 8 % than at 4 % concentration, whereas maltose in the same range was fermented by the sample at a uniform rate.

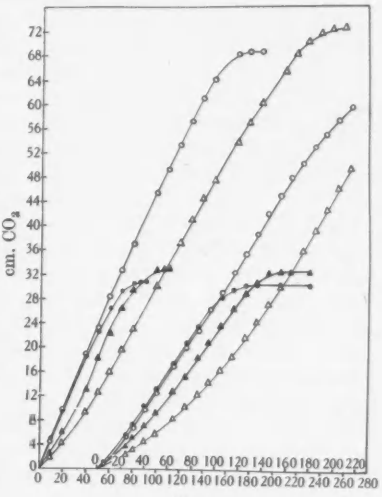


Fig. 4. Fermentation of maltose and glucose by exceptional lot of brewer's yeast. Solutions, 4·0 ml. containing citrate-phosphate buffer *p*H 6·8 prepared according to Willstätter & Bamann [1926] and at 20-fold final dilution, 10 % Richon brewer's yeast, and designated concentrations of sugar. Left hand curves, fermentation by cells fresh from brewery; right hand curves, fermentation by same cells after storage on ice for 2 weeks. (1, ●—●) maltose 4 %, (2, ▲—▲) glucose 4 %, (3, ○—○) maltose 8 %, (4, △—△) glucose 8 %.

V_m/V_g fermentation ratios greater than unity have been reported previously by Trautwein & Wassermann [1929], Yamasaki [1930] and by Sobotka & Holzman [1934]. Working with variously aged samples of Richon yeast and in media of injuriously high buffer salt concentration, e.g. in undiluted citrate-phosphate prepared according to Willstätter & Bamann [1926], the present authors have observed V_m/V_g values consider-

ably greater than unity with fair regularity [Leibowitz & Hestrin, 1940; Hestrin, 1941]. The effect on fermentation under these conditions of ageing and of substrate concentration was qualitatively as noted in Fig. 4 for the 'exceptional yeast lot' in relatively low salt medium, and need not be reported separately.

The significance of these findings for direct fermentation theory remains to be considered. The interpretation of the evidence to hand is, however, problematic, and complicated by the following considerations:

(1) V_m/V_g ratios greater than unity have been observed in buffer of *p*H 6·8. At this *p*H, however, the activity of maltozymase, at least in baker's yeast, appears to be completely suppressed.

(2) In all the experiments where maltose fermented more rapidly than glucose, increased concentration of glucose led to a lowered rate of glucolysis. It is therefore conceivable that V_m/V_g values greater than unity were only obtained here because in the presence of excess of glucose the full glucolytic ability of the yeast failed to come into play.

Analogous possibilities must also be considered in connexion with other findings of superior disaccharide fermentability, reported in the literature (Willstätter & Oppenheimer [1921] and Hestrin [1941] for lactose; Wright [1936] for sucrose; Myrbäck & Örtenblad [1937] and O'Connor [1940] for trehalose).

(*c*) p*H relations*. Experiments comparing the dependence of maltose fermentation on *p*H by brewer's and baker's yeast with that of methyl α-glucoside fermentation by brewer's yeast and all three with the known *p*H function of dissolved maltase are summarized in Fig. 3. It is evident from the figure that the *p*H-activity curves of cell-free and cell-bound maltase (=α-glucosidase) are essentially similar (curves III and IV respectively). The relatively slower drop in activity of cell-bound maltase at values below *p*H 6·0 is probably only apparent and due to a modification of intracellular *p*H in this range by cell-formed buffer substances.

The presence of active maltase in fresh cells of Richon yeast is confirmed by the positive fermentation test of this yeast to methyl α-glucoside. This conclusion further suggests a ready explanation of the difference in the *p*H-activity curves of maltose fermentation by baker's and brewer's yeasts respectively (curves II and V). It is to be expected that in the presence of α-glucosidase maltose will be fermented at *p*H 7·0 which is in the optimum range for this enzyme. The experiment summarized in curve V shows that this is indeed the case. The true *p*H dependence of *direct* maltose fermentation is presumably masked in the case of brewer's yeast by the activity in the neutral range of cell maltase.

Discussion

Experiments reported in the present and in previous investigations [Leibowitz & Hestrin, 1939; Schultz & Atkin, 1939; 1940; Kluyver & Custers, 1940] confirm that under suitable conditions maltose fermentation by yeast may be selectively inhibited or accelerated by agents which are without known similar or comparable influence on either glucozymase or maltase. The differential effects of certain sugars, hydrogen ion concentration, and oxygen on the fermentation of maltose and glucose by baker's yeast are compared in the following table.

	Effect on fermentation of	
Agent	Glucose	Maltose
Maltose	None	Inhibition (variable)*
Methyl α-glucoside	None	Inhibition
Zymohexose (traces)	None	Activation
O_2	Inhibition	Activation†
*p*H 4·5	Optimum	Optimum
*p*H 7·0	Optimum	Complete inhibition

* In concentration range providing adequate amount of substrate.
† Schultz, Atkins & Frey [1940].

Since the listed reagents affect maltose fermentation in an essentially different manner from glucose fermentation it is obviously necessary to assume that maltose fermentation involves a specific rate-limiting component which is absent from glucose fermentation. The question arises whether the specific component is maltase. Discussion of this question must be preceded, however, by consideration of the possibility that the different effects noted are manifestations of specific effects on the permeability apparatus involved in maltose absorption, rather than effects on the fermentation enzymes themselves.

The suggestion has been entertained that O_2 and glucose influence the rate of gas evolution from maltose by affecting the rate at which maltose diffuses into the cell [Schultz *et al.* 1939; 1940]. Improved permeability could only affect the gassing rate, however, where the rate of diffusion is the limiting factor. Available evidence weighs against this conclusion. The measurements of Sobotka *et al.* [1936] show that maltose diffuses into baker's yeast at least one-half as rapidly as glucose. But maltose is frequently fermented by glucose-activable yeast with a velocity considerably below half that of glucose. The limiting factor cannot therefore be the rate of diffusion. This conclusion is further strengthened by the observation that maltose fermentation has a high temperature coefficient [Slator, 1908] and also by kinetic data advanced above. It is to be expected on the view that permeation is the rate-limiting process that increase in the substrate concentration would lead to increased permeation, a resultant increase in gassing rate and a corresponding lessening of the yeast's susceptibility to activation by glucose. None of these predictions is realized in practice (cf. Tables 1 and 3, and Fig. 2). It seems correct to conclude therefore that the zymohexoses and O_2 do not affect maltose fermentation through their action on permeability.

The above conclusion may be further extended to the substrate-specific retardant effect of methyl α-glucoside on maltose fermentation. If permeability were rate-limiting for maltose fermentation in the presence of a given inhibitor, activators of reaction steps subsequent to the diffusion process should fail to affect the gassing rate. It has been shown, however, that glucose can activate maltose fermentation both in the presence and absence of methyl α-glucoside (Table 4). Since it has been concluded that glucose acts on the fermentation sequence at a stage subsequent to the diffusion process it becomes necessary to assume also that methyl α-glucoside acts on maltose fermentation at a stage identical with or subsequent to that acted on by glucose, i.e. a stage other than that of the permeation process.

The view that hydrolysis by maltase is the rate-limiting component in the fermentation of maltose by baker's yeast necessitates the assumption that zymohexose, O_2, methyl α-glucoside, and maltose in high concentration affect the rate at which maltose is fermented through specific actions on cell maltase. None of these substances affects the activity of maltase in solution in a manner comparable to their action on maltose fermentation. Glucose which stimulates maltose fermentation is itself formed from maltose by maltase but its action on maltase is that of an inhibitor [Kuhn, 1923]. Stimulation of maltase activity by O_2 has never been demonstrated. Maltose and methyl α-glucoside, again, are substrates of yeast maltase. It has been shown that increase of the maltose concentration within a wide range does not inhibit but leads to an increase in the rate at which glucose is liberated by maltase [Willstätter, Kuhn & Sobotka, 1924]. The view that methyl α-glucoside affects maltose fermentation by competing for maltase enzyme with maltose is superficially attractive but fails to explain an important characteristic of the inhibition: the virtual absence of inhibitory response when methyl α-glucoside is added after maltose fermentation has already fully set in (Table 5). It is to be expected, moreover, in this view that methyl α-glucoside would itself be split by maltase and hence be slowly fermented. The fresh baker's yeast failed, however, under the conditions of the experiments to ferment methyl α-glucoside. The absence of such fermentation must be regarded as evidence that the baker's yeast cells contained no active maltase (=α-glucosidase).

Further support for the view that hydrolysis by maltase is not the limiting factor of, or indeed necessary to, maltose breakdown is provided by consideration of the *p*H-activity curves of maltose, glucose, and methyl α-glucoside fermentation respectively. Fig. 3 shows that our baker's yeast though able to ferment maltose in the tested *p*H range 3·0–6·0 is normally unable in adequately buffered medium to ferment this disaccharide at *p*H 7·0, the known optimum *p*H of both dissolved and cell-bound maltase. Glucose is fermented at *p*H 7·0 with optimum velocity.

The specific activating effect of zymohexoses and the retarding action of saccharides such as methyl α-glucoside on maltolysis becomes comprehensible, on the other hand, if the view is accepted that maltose fermentation involves the specific participation of activators which are formed in the cell during and by the breakdown of fermentable sugar [Leibowitz & Hestrin, 1939]. The activation, it may be noted, persists long after the last trace of glucose has been exhausted by fermentation. Its dependence on a substance formed during the fermentative breakdown of hexose rather than on hexose as such is confirmed by the finding that only fermentable hexoses but not galactose activate maltose fermentation (Table 6).

The effect of glucose is complex: in weakly buffered media near neutral *p*H, glucose fermentation causes a shift in *p*H to greater acidity [Mirski & Wertheimer, 1939] and this favours maltose fermentation. There remains, however, at acid *p*H a residual activation effect due to glucose [Schultz & Atkin, 1939]. This is manifested at all maltose concentrations through a curtailment of the induction period which precedes the onset of gas evolution, but at high maltose concentration may also be manifested through an increase of the stationary gassing rate ultimately attained (Fig. 2).

Little is known concerning the nature of the specific catalysts of maltolysis. The discovery that O_2 plays an activating role in maltose fermentation [Schultz *et al.* 1940; Kluyver & Custers, 1940] may throw light on this subject. Schultz *et al.* [1940] mention that in the presence of traces of glucose the effect of O_2 is masked. It is therefore possible that glucose acts functionally as a substitute for O_2. It has been shown that both O_2 and glucose exert a depressant effect on the concentration of phosphate in the yeast cell [Macfarlane, 1936; Johnson, 1941]. The possibility that their common stimulative action on maltolysis is mediated through an effect on phosphate will therefore deserve investigation.

A further weapon for the elucidation of the catalyst complex involved in maltolysis is provided by methyl α-glucoside. This reagent appears to constitute a specific retardant of maltose fermentation by baker's yeast. Its effect is not given, moreover, by methyl β-glucoside or by methanol. It has been shown that when methyl α-glucoside is added to the maltose-yeast mixture after the beginning of gas evolution its inhibiting efficiency is largely abolished (Table 5). Cells fresh from a previous maltolysis are relatively indifferent to inhibition by methyl α-glucoside presumably because such cells already possess a ready store of the catalyst system whose formation would have been retarded in the presence of methyl α-glucoside. It seems correct to conclude therefore that methyl α-glucoside acts not as a retardant of the main cycle of maltolysis but rather as a specific retardant of a catalyst-forming side-reaction. A possible role for O_2 in this side-reaction is suggested by the observation that methyl α-glucoside acts also as a retardant on oxidation systems [Johnson & Anderson, 1938].

Methyl α-glucoside, which is also a sucrase inhibitor, slightly retarded raffinose fermentation but was without noticeable effect on sucrose fermentation by a baker's yeast. It therefore seems correct to conclude that invertase action was rate-limiting in raffinose fermentation but not in sucrose fermentation by this baker's yeast.

Work on the nature of maltolysis must further centre in future on the identification of the metabolite in the formation of which the different reaction paths of maltolysis and glucolysis are assumed to merge. It has been suggested previously, in view of the findings

of Willstätter & Rohdewald [1937; 1940], that this metabolite is glycogen [Leibowitz & Hestrin, 1939]. The finding that certain preparations of phosphorylase may act on maltose as well as on higher polysaccharides though not on glucose [Hanes, 1940; cf. however, Cori, Colowick & Cori, 1938] raises the further possibility that the unnamed first common metabolite of glucolysis and maltolysis is glucose-1-phosphate. The first step in maltolysis would on this view be a phosphorylysis of the maltose molecule.

The relative importance of the maltase-glucozymase complex and of maltozymase, respectively, in maltose fermentation by yeast is obviously dependent on a variety of factors including *p*H and temperature [cf. Leibowitz & Hestrin, 1939]. The two systems are not, however, necessarily of the nature of competitors, but act each in separate though overlapping *p*H ranges. Maltozymase, as is shown by Fig. 3, is exclusively responsible for maltose fermentation at *p*H values below 4·0; a maltase-glucozymase system is responsible for any maltose fermentation at *p*H values above 6·8.

The failure of a yeast sample to ferment maltose at *p*H 7·0 is valid evidence that under the conditions of the test maltase is not actively present in the cells, but does not constitute proof that preparations of the cells will be inactive to maltose. Experiments summarized in Table 7, show that yeast cells which failed to ferment maltose at *p*H 7·0, and methyl α-glucoside at any *p*H, acquire ability to ferment these substrates at neutral *p*H after being dried. This finding suggests that maltase may exist in the cell in an inactive form. This maltasogen is presumably convertible into active maltase through dissolution of the cell structure by drying or autolysis (cf Table 8). The much discussed unreliability of in vitro analysis as a method of determining maltase activity in vivo is thus further confirmed but in an unexpected direction. It has been widely accepted, hitherto, that absence of a given enzyme in cell preparations does not constitute proof of its absence in life. The possibility has also to be considered, however, that an enzyme may become active in vitro which was inactive in the living cell.

Summary

Experiments are reported dealing with the relative role and nature of the direct and indirect enzyme mechanisms of maltose fermentation in yeast.

A. *Baker's yeast*

(1) Different agents may selectively inhibit or stimulate maltose fermentation by baker's yeast without similar effect on glucolysis or maltase activity.

(*a*) Maltose fermentation was stimulated by any of the zymohexoses but not by galactose.

(*b*) Methyl α-glucoside is a powerful substrate-specific retardant of maltose fermentation by baker's yeast. The inhibition by methyl α-glucoside is completely reversible. Its intensity increases with increasing concentration of glucoside. Methyl β-glucoside and methanol in equivalent concentration did not inhibit maltose fermentation.

(*c*) High concentrations of maltose may inhibit the rate at which maltose is fermented by aged baker's yeast but do not similarly affect glucose fermentation or maltase activity in vitro. Addition of glucose traces abolished the retardant effect of high maltose concentration.

(*d*) The *p*H-activity curve of cell-bound maltase was largely identical with that of cell-free maltase, distinct from that of glucozymase, and actually opposite in trend to the *p*H-activity curve of maltose fermentation.

(*e*) Maltose is vigorously fermented by living baker's yeast at acid *p*H values which preclude hydrolysis by either cell-free or cell-bound maltase, but was not fermented at all by the same cells at neutral *p*H, which is optimal for maltase.

The conclusion follows that maltose fermentation by baker's yeast is direct.

(2) The view is advanced that the maltozymase complex of yeast involves specific maltose fermentation catalysts and that these are generally formed in the cell during maltose fermentation.

(*a*) Methyl α-glucoside only retarded maltolysis when added to the fermentation mixture before maltose fermentation had become vigorous.

(*b*) Traces of glucose markedly curtailed, but never entirely abolished, the induction period preceding the onset of the stationary phase of maltose fermentation.

(3) Baker's yeast cells failed to ferment maltose at neutral *p*H, and methyl α-glucoside at any *p*H, but acquired ability to ferment these substrates at *p*H 7·0 when dried. The fresh cells yielded active typical maltase on autolysis. It seems necessary to conclude that these yeast cells may contain maltase in inactive forms (maltase zymogens).

B. *Brewer's yeast*

(4) Maltose fermentation by brewer's yeast differed in several respects from that by baker's yeast.

(*a*) Ordinarily samples of brewer's yeast fermented maltose and glucose at an equal rate. As expected, maltose fermentation was here found insusceptible of inhibition by methyl α-glucoside or of activation by traces of hexose.

(*b*) At high substrate concentration, maltose was in certain cases fermented more rapidly than glucose.

(*c*) Maltose fermentation by brewer's yeast occurs at neutral as well as at acid *p*H (3·0–7·0). The same cells ferment methyl α-glucoside, vigorously in the neutral range, but not at all below *p*H 4·0. They may be concluded to contain active maltase.

It is suggested that at acid reaction, brewer's yeast ferments maltose by means of a direct fermentation mechanism (maltozymase) and that an indirect fermentation mechanism—maltase + glucozymase—is solely or largely responsible for fermentation of maltose in the neutral range.

REFERENCES

Beijerinck, M. W. [1895]. *Zbl. Bakt.* Abt. II, **1**, 221.
Blish, M. J. & Sandstedt, R. [1937]. *J. biol. Chem.* **118**, 765.
Cori, G. J., Colowick, S. P. & Cori C. F. [1938]. *J. biol. Chem.* **123**, 375.
Deere, C. J., Dulaney, A. D. & Michelson, I. D. [1939]. *J. Bact.* **37**, 355.
Genevois, L. [1937]. *Ann. des Ferment.* **3**, 600.
Hanes, C. S. [1940]. *Proc. roy. Soc.* B, **128**, 421; **129**, 174.
Hestrin, S. [1941]. The decomposition of maltose by micro-organism, Jerusalem. Dissertation.
Hofmann, E. [1934]. *Biochem. Z.* **272**, 417.
Hopkins, R. H. & Roberts, R. H. [1935]. *Biochem. J.* **29**, 919.
Johnson, F. H. [1941]. *Science*, **94**, 200.
Johnson, F. H. & Anderson, R. S. [1938]. *J. cell. comp. Physiol.* **12**, 273, 281.
Kluyver, A. J. & Custers, M. T. J. [1940]. *Antony v. Leeuwenhoek*, **6**, 121.
Kuhn, R. [1923]. *Hoppe-Seyl. Z.* **127**, 234.
Leibowitz, J. & Hestrin, S. [1939]. *Enzymologia*, **6**, 15.
—— —— [1940]. *Nature, Lond.*, **145**, 671.
Macfarlane, M. C. [1936]. *Biochem. J.* **30**, 1369.
Michaelis, L. & Pechstein [1914]. *Biochem. Z.* **60**, 79.
Mirski, A. & Wertheimer, E. [1939]. *Enzymologia*, **7**, 65.
Myrbäck, K. & Örtenblad, B. [1936]. *Biochem. Z.* **288**, 329.
—— —— [1937]. *Biochem. Z.* **291**, 61; **292**, 230.
Neuberg, C. & Hofmann, E. [1932]. *Biochem. Z.* **256**, 450.
Nord, F. F. & Weichherz [1929]. *Z. Elektrochem.* **35**, 612.
O'Connor, R. C. [1940]. *Biochem. J.* **34**, 1008.
Schultz, A. S. & Atkin, L. [1939]. *J. Amer. chem. Soc.* **61**, 291.
Schultz, A. S., Atkin, L. & Frey, C. N. [1940]. *J. Amer. chem. Soc.* **62**, 2271.
Slator, A. [1908]. *J. chem. Soc.* (*Trans.*), **93**, 217.
Sobotka, H. & Holzman, M. [1934]. *Biochem. J.* **28**, 734.

Sobotka, H., Holzman, M. & Reiner, M. [1936]. *Biochem. J.* **30**, 933.
Trautwein, K. & Wassermann, J. [1929]. *Biochem. Z.* **215**, 293.
Willstätter, R. & Bamann, E. [1926]. *Hoppe-Seyl. Z.* **151**, 242; **152**, 202.
Willstätter, R., Kuhn, R. & Sobotka, H. [1924]. *Hoppe-Seyl. Z.* **134**, 224.
Willstätter, R. & Oppenheimer, L. C. [1921]. *Hoppe-Seyl. Z.* **118**, 168.
Willstätter, R. & Rohdewald, M. [1937]. *Hoppe-Seyl. Z.* **247**, 269.
—— —— [1940]. *Enzymologia*, **8**, 1.
Willstätter, R. & Steibelt, W. [1921]. *Hoppe-Seyl. Z.* **115**, 219.
Wright, H. D. [1936]. *J. Path. Bact.* **42**, 31; **43**, 487.
Yamasaki, I. [1930]. *Biochem. J.* **228**, 127.

89. The Digestibility of Wheat Straw and Wheat-Straw Pulp

By **William Swan Ferguson**, *From Imperial Chemical Industries' Agricultural Research Station, Jealott's Hill, Bracknell, Berks*

(*Received 3 August 1942*)

The digestion of straw constituents and cellulose by ruminants was the subject of a recent paper by McAnally [1942]. By means of in vitro and also in vivo experiments, using a sheep with a rumen fistula, she examined the rate of digestion of straw, straw constituents and cellulose. In the in vivo tests small quantities (0·5 g.) of the materials were tied in small silk squares and suspended in the sheep's rumen. McAnally states: 'it is probable that the conditions of digestion in the bag were not as favourable as in the rumen, so that the observed rates of digestion must be regarded as minimal.' The results showed that straw fractions such as cellulose and hemicelluloses were more digestible when separated than when fed in straw form.

A proportion of the straw resists digestion regardless of time, but McAnally thinks that too rapid progress through the rumen can cause considerable wastage and suggests some form of predigestion in vitro to eliminate this.

The digestibility of straw soaked for 24 hr. in 1·5% KOH and then thoroughly washed was also investigated, and a marked advantage over untreated straw was obtained. During the first 2 days in the rumen the digestion of the treated straw was no greater than that of untreated straw, and it is concluded that 'wastage through too rapid a passage out of the rumen may not be avoided by the alkali pretreatment'. The digestibility of the lignin in the alkali-treated straw was 71% as compared with 30% in the untreated straw.

Investigations on the alkali treatment of straws to increase their feeding value were commenced at Jealott's Hill in 1938, and in 1940, in view of the inadequacy of the conventional foodstuffs analysis, a trial was made to determine the digestibility of the true straw constituents. The results showed that the digestibility of the cellulose of alkali-treated straw was very high and they suggested that wastage, if any, by too rapid progress through the rumen must be quite small. They also suggest that the technique of McAnally does result in slower rates of digestion than that which occurs normally in the rumen.

The data presented here give only the fate of the various straw fractions during passage through the animal, or the total digestibility as understood in agricultural circles.

Experimental

A uniform sample of good quality wheat straw was used throughout the trials. It was chopped to half-inch lengths and well mixed.

Alkali treatment. The chopped straw was immersed in 1·5% NaOH for 22 hr. at 10–15°, using 10 times the straw weight of solution. At the end of the time, the straw was removed from the liquor and after draining for 30 min. was thoroughly washed with water to remove the excess NaOH. The straw lost 20% of its dry weight in this treatment. For convenience the resulting material was called 'straw pulp'.

Composition. The untreated straw and the straw pulp were dried at 98°, ground and analysed by the standard feedingstuffs methods, and also in greater detail. Cellulose was determined by the method of Norman & Jenkins [1933] and also by that of Kürschner & Hanak [1930] as used by Crampton & Maynard [1938], the cellulosan fraction of the crude cellulose being determined by distillation with 12% HCl. Lignin was determined by the method of Ritter, Seborg & Mitchell [1932] as modified by Norman [1934]. The

total furfuraldehyde yield was obtained by distillation with 12% HCl and subsequent precipitation as the phloroglucide.

Digestibility. Digestibility trials were carried out on sheep. Six sheep were used in each trial, the same sheep being used for both materials. The rations consisted of 600–700 g. of straw dry matter and 100 g. casein to supply protein. Rock salt and steamed bone flour were also provided. The straw pulp was naturally fed in the wet state. After a preliminary period of 4 days the faeces were collected daily for 10 days.

Results

Composition

The standard feedingstuffs analysis is not given here in detail and it need only be stated that the crude fibre fraction which was 42·4% of the dry matter in the untreated straw and 51·5% of the dry matter in the straw pulp consisted of 90–93% crude cellulose and 7–10% lignin. The more detailed composition of the straws is given in Table 1.

Table 1. *Composition of untreated straw and straw pulp (as percentage dry matter)*

	Untreated straw	Straw pulp		Untreated straw	Straw pulp
Ether extract	1·05	1·13	True cellulose*	35·28	43·78
Crude protein	2·78	2·02	Pentosans in cellulose	18·93	20·09
Ash	7·47	6·94	Furfuraldehyde	5·73	6·59
Lignin	15·10	13·64	Other carbohydrates	13·66	5·81
				100·00	100·00

* Norman & Jenkins's true cellulose.

The analysis accounts for most of the dry matter in the straws. In the untreated straw 13·7% was undetermined and has been called 'other carbohydrates'. This fraction presumably consists mainly of carbohydrate material of more simple constitution than cellulose, and was evidently partly soluble in the NaOH. Only traces of reducing sugars were present. The furfuraldehyde fraction represents the hemicelluloses excluding the cellulosan fraction of the cellulose and some doubt exists as to the correct method of expressing the yield. Some workers convert the yield of furfuraldehyde into pentosans which increases the value by about 70%, but Norman [1937] thinks the more accurate way is to leave the fraction expressed as furfuraldehyde.

The most noticeable differences between the straws are the higher cellulose and lower 'other carbohydrate' contents of the straw pulp.

The analytical data clearly showed the mixed nature of the crude fibre and N-free extractives of the standard feedingstuffs analysis. The crude fibre consists mainly of cellulose from which a considerable part of the cellulosan has been removed, and some lignin. The N-free extractives are made up of part of the lignin, part of the cellulosan, the furfuraldehyde-yielding substances and 'other carbohydrates'.

The cellulose contents of the straw were also determined by the method of Kürschner & Hanak [1930]. It will be seen from Table 2 that this method gives a lower yield of crude cellulose than Norman & Jenkins's [1933] method, owing to the loss of a considerable part of the cellulosan fraction.

Table 2. *Cellulose contents of untreated straw and straw pulp. Comparison of methods (as percentage dry matter)*

	Untreated straw		Straw pulp	
	N. and J.*	K. and H.†	N. and J.	K. and H.
Crude cellulose	54·21	39·45	63·87	52·18
True cellulose	35·28	35·00	43·78	43·81
Pentosan	18·93	4·45	20·09	8·37

* Norman & Jenkins's method. † Kürschner & Hanak's method.

Digestibility

The faeces of the sheep were analysed in the same way as the straws. It is interesting to note that the crude fibre in the faeces of the sheep receiving untreated straw consisted of 86% cellulose (almost entirely true cellulose) and 14% lignin, whereas in the case of the straw-pulp faeces the crude fibre contained 60% true cellulose and 40% lignin. These proportions are quite different from those found in the fibres of the original materials fed and show the unreliability of the crude fibre digestibility figures. It follows, therefore, that the digestibility value for the N-free extractives are also unreliable.

The average digestibility coefficients are given in Table 3 and the values for the individual sheep in the Appendix. The digestibility of the crude protein was ignored since this constituent forms a very small part of the whole.

Table 3. *Average digestibility coefficients of the straw constituents*

Constituent	Untreated straw	Straw pulp	Constituent	Untreated straw	Straw pulp
Dry matter	47·8	63·8	Lignin	10·2	−0·5
Organic matter	51·8	68·5	Crude cellulose	62·4	87·7
			True cellulose	62·1	86·7
Ether extract	43·7	40·9	Pentosans in cellulose	63·2	89·7
Fibre	60·3	81·0	Furfuraldehyde	51·2	84·2
N-free extractives	46·8	63·1	Other carbohydrates	67·0	76·9

It is clear that the alkali treatment raised the digestibility of most of the constituents. The digestibilities of the true cellulose and pentosans in cellulose run parallel and therefore, the sum of these constituents, namely the crude cellulose, only need be considered. Its digestibility was raised from 62·4 to 87·7% by the treatment. The furfuraldehyde-yielding substances also increased markedly in digestibility. The 'other carbohydrates' showed a less marked increase, as might be expected from the nature of this fraction. The average digestibility of the lignin in the untreated straw was 10·2%, the range in the individual sheep being 4·4–16·6%. In the straw pulp a figure of −0·5%, or total indigestibility was obtained, the range being −9·1 to 6·4%. It can only be assumed that the treatment with alkali removed a part of the lignin which possessed some slight digestibility. It must be remembered, however, that the determination of lignin cannot be considered altogether reliable and appreciable errors could be introduced in this way.

Discussion

During the passage of straw pulp through the sheep, approximately 70% of the dry matter and 90% of the cellulose were digested. This was quite efficient digestion and it occurred in a probable maximum of 3½ days. It seems unlikely that the food remained in the rumen for more than 2–2½ days. It is generally thought that in ruminants practically all the cellulose digestion or fermentation occurs in the rumen and the work of Trautmann & Asher [1939] certainly confirms this. The present work therefore cannot support McAnally's view that wastage by too rapid passage through the rumen might occur with straw pulp. It is interesting to note that McAnally obtained a figure for the digestibility of true cellulose of 88·8% by suspending straw pulp in the rumen for 7 days. This is very close to the value of 86·7% obtained for complete digestion in the present work. This also suggests that McAnally's technique gives a rate of digestion which is considerably slower than that occurring normally in the rumen.

The lignin appears quite indigestible in straw pulp and this does not agree with McAnally's findings. She obtained 70% digestibility of this constituent in the rumen and two possible explanations of the difference might be put forward. Either the lignin is considerably digested or rendered soluble in the rumen and then later deposited in

some other part of the alimentary tract, or the difficulty in dealing with small quantities of material in the rumen fistula has introduced some error.

The digestibility data, which are typical of many obtained at Jealott's Hill, clearly show the benefit derived from the alkali treatment of straw. The increase in digestibility has the effect of approximately doubling the feeding value of good wheat straw to the ruminant.

Summary

The digestibilities of straw constituents in wheat straw and wheat straw treated with sodium hydroxide solution (straw pulp) have been determined on sheep.

The alkali treatment results in a marked increase in the digestibility of all constituents except lignin. The lignin was totally indigestible.

Owing to the high digestibility of the major straw-pulp constituents, it is unlikely that any wastage occurs owing to too rapid passage of the material through the rumen.

The inadequacy of the conventional feedingstuffs analysis is discussed briefly.

The author wishes to thank Dr S. J. Watson for his interest in the work and the Directors of Imperial Chemical Industries Ltd., for permission to publish the results.

REFERENCES

Crampton, G. W. & Maynard, L. A. [1938]. *J. Nutrit.* **15**, 383.
Kürschner, K. & Hanak, A. [1930]. *Z. Untersuch. Lebensmitt.* **59**, 484.
McAnally, R. A. [1942]. *Biochem. J.* **36**, 392.
Norman, A. G. [1934]. *Biochem. J.* **28**, 2147.
—— [1937]. *The Biochemistry of Cellulose, the Polyuronides, Lignin, etc.* Oxford University Press.
Norman, A. G. & Jenkins, S. H. [1933]. *Biochem. J.* **27**, 818.
Ritter, G. J., Seborg, R. M. & Mitchell, R. L. [1932]. *Industr. Engng. Chem.* (Anal. ed.), **4**, 202.
Trautmann, A. & Asher, T. [1930]. *Z. Tierernähr. Futtermittelk.* **3**, 45.

APPENDIX

Digestibility coefficients

Sheep ...	7	8	9	10	11	12	Average
Untreated straw:							
Dry matter	43·7	47·1	47·0	47·5	49·0	52·4	47·8
Organic matter	48·0	50·7	51·1	51·0	53·3	56·6	51·8
Ether extract	36·4	59·3	43·5	47·7	34·9	40·2	43·7
Crude fibre	57·8	58·6	55·5	60·2	63·1	66·3	60·3
N-free extractives	42·3	46·2	46·9	45·7	48·0	51·6	46·8
Lignin	4·4	13·9	10·5	6·0	9·8	16·6	10·2
Crude cellulose	60·0	60·4	59·9	62·4	64·9	67·0	62·4
True cellulose	59·9	59·6	60·2	62·2	63·9	66·5	62·1
Pentosans in cellulose	60·3	62·0	59·4	62·9	66·7	67·9	63·2
Furfuraldehyde	45·3	55·3	56·5	50·0	45·2	54·8	51·2
Other carbohydrates	60·6	60·4	69·7	66·8	71·2	73·3	67·0
Straw pulp:							
Dry matter	66·5	61·1	61·5	60·0	67·3	66·1	63·8
Organic matter	71·4	65·3	65·8	64·9	72·6	70·9	68·5
Ether extract	49·2	40·3	25·6	33·9	50·0	46·1	40·9
Crude fibre	83·6	77·0	80·2	78·3	83·9	82·9	81·0
N-free extractives	66·1	61·9	58·9	59·2	67·4	65·1	63·1
Lignin	4·4	−1·1	−9·1	−6·1	6·4	2·8	−0·5
Crude cellulose	90·6	84·1	86·0	85·0	90·6	89·7	87·7
True cellulose	89·7	82·5	85·0	84·3	89·9	88·7	86·7
Pentosans in cellulose	92·6	87·1	88·0	86·5	92·3	91·8	89·7
Furfuraldehyde	84·3	81·2	83·8	85·4	85·2	85·0	84·2
Other carbohydrates	75·9	77·3	81·4	68·8	81·6	76·2	76·9

90. A Steam Distillation Apparatus Suitable for Micro-Kjeldahl Analysis

By **Roy Markham**, *Plant Virus Research Station and Molteno Institute, Cambridge*

(*Received 30 July 1942*)

The apparatus to be described is a simple and efficient steam distillation apparatus. Originally designed for the distillation of volatile fatty acids, it has proved very convenient for micro-Kjeldahl analyses.

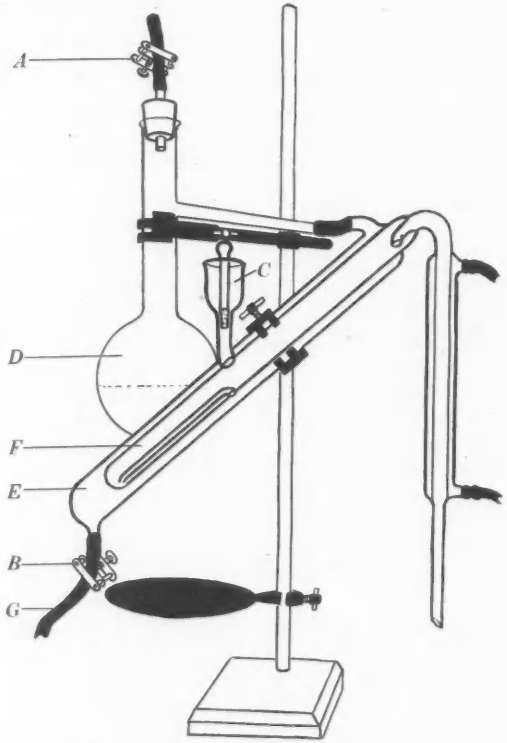


Fig. 1. The steam-distillation apparatus with steam generator and connexions, mounted on a retort stand.

Basically similar to the Pregl apparatus with its many modifications, it differs in some structural details, the result being a compact apparatus with a minimum of rubber connexions, and, as it may be mounted on a single retort stand, it takes up very little bench space.

Fig. 1 shows the apparatus,* which is blown from 'Phoenix' resistance glass.

* Obtainable from Messrs W. G. Flaig and Sons, Ltd., 39 Waterloo Road, Cricklewood, London, N.W. 2.

Operation

The sample to be analysed, containing 0·02–0·15 mg. N, is incinerated in the usual way.

The apparatus is steamed out before operation and the screw clips *A* and *B* are opened. The digest is washed into the apparatus quantitatively with 5–10 ml. distilled water through the funnel *C*, which is fitted with a ground-in stopper. The stopper is replaced and 2 ml. 40 % NaOH are poured into the funnel.

The burner is placed under the distillation flask *D*, which serves as a steam generator and is connected to the still by a short rubber connexion. When steam is evolved, screw clip *A* is closed and steam passes into the steam jacket *E*, where it condenses until the liquid in *F* is at 100° when live steam escapes from the tube *G* which leads into a sink.

Screw-clip *B* is then closed and the stopper in the funnel *C* is lifted slightly, letting the strong soda flow into the digest.

Distillation then proceeds, preferably at a rate of 8–10 ml./min. (the condenser is capable of condensing some 10–12 ml./min.). The distillate is collected in a sufficient quantity of dilute acid. When dealing with such small quantities of ammonia it is not desirable to dip the tip of the condenser into the acid. No loss can be detected owing to the small volatility of the dilute ammonia, and the necessity for washing the condenser tip and collecting a larger quantity of distillate is avoided. Practically complete recovery of the ammonia is obtained in 8 ml. of distillate, and it is unnecessary and undesirable to collect more than 10 ml.

When the distillation is complete, the burner is removed and the liquid in *F* flushes automatically into *E*. Water is poured into *C* and washes through into *E*. Clips *B* and *A* are opened, the fluid in *E* runs down the drain and the apparatus is ready for another distillation, the whole operation taking 1–1½ min.

Using *N*/100 HCl with methyl red-methylene blue as indicator and titrating with *N*/100 baryta in a volume of 8 ml., it is possible to judge the end-point of the titration to less than 1 % in estimating 0·02 mg. N, and, using 0·03–0·05 mg. N, it is easy to obtain duplicates agreeing to less than 1 % without taking any extreme precautions.

When using the apparatus with 0·5 mg. N or more it is recommended that the usual technique of immersing the condenser tip and collecting a larger quantity of distillate should be adopted.

I should like to express my gratitude to my friends E. Benton (now serving with H.M. Forces in the Middle East) and H. W. Hall, technicians in the Biochemical Department, Cambridge, for their assistance.

Summary

A highly efficient steam-jacketed steam distillation apparatus is described.

Details are given of its application to the estimation of quantities of N down to 0·02 mg.

91. The Physiological Properties of Vitamin A

1. A Specific Effect upon Body Weight and Body Composition in the Albino Rat

By **Jean M. Patterson**, **E. W. McHenry** and **W. A. Crandall**, *From the School of Hygiene, University of Toronto and the Laboratory of Hygiene, Department of Pensions and National Health, Ottawa*

(*Received 3 September 1942*)

In the case of most of the vitamins a deficiency causes a restriction in food consumption. The question has frequently been raised as to whether observed results in vitamin experiments are due directly to a deficiency of a vitamin or to the lowered food intake. The use of isocaloric feeding has given significant results in the case of several of the vitamins but has seldom been reported for vitamin A. Sampson & Korenchevsky [1932] found that pair-fed rats receiving vitamin A were heavier and had more body fat than animals similar in food intake but not receiving the vitamin. Green [1934] reported that the loss in weight in rats deprived of vitamin A was not significantly greater than in animals with a restricted food supply and which received the vitamin. In view of the conflicting evidence we decided to study the possible specific effect of vitamin A upon body weight. Six series of observations in two laboratories, in different seasons, have given results in complete agreement. Results of only one will be presented, and data on body weight and composition will be included.

Experimental methods

Albino rats of the Wistar strain, reared in the Connaught Laboratories' colony or in the Laboratory of Hygiene, were used at the age of 3–4 weeks with an initial weight of about 50 g. They were housed in individual screen-bottom cages at room temperature of 72–74° F. Water was supplied *ad lib.* The basal diet was that recommended by Coward [1938] for the biological assay of vitamin A; it had the following composition:

Casein, Labco vitamin-free	15%
Dextrinized rice starch	73%
Brewers' yeast, Mead Johnson	8%
Salt mixture, Steenbock	4%

As a source of vitamin A, a solution of carotene in corn oil was used in some experiments and in others a cod liver oil concentrate. Results from both sources of vitamin A activity were similar. In each case all rats received the same amount of vitamin D (10 i.u. per week), sufficient irradiated ergosterol being added for that purpose.

In each series of experiments 3 groups of animals were employed, the corresponding numbers of the groups being of the same sex and approximately the same weight. Each group contained at least 10 animals. In each series the 'normal' group received the basal diet *ad lib.* plus a daily supplement of 5 i.u. vitamin A. The 'basal' group was given the basal diet *ad lib.*, and the 'paired' group received the daily supplement of 5 i.u. vitamin A and an amount of food equal to that consumed by the corresponding animal in the basal group on the previous day. The animals were weighed 3 times weekly during the first 4 weeks and daily thereafter. Daily records of food consumption were kept.

In the series reported, determinations of body composition and analyses of food and faeces were made. During the first 4 weeks on the deficient diet, animals in the basal group continued to gain in weight and no changes in appetite were observed. After 4 weeks it was likely that body stores of the vitamin were considerably depleted, since the

basal animals then showed diminishing appetite and a failure to increase in weight. For this reason analyses of body composition were made at the end of the first 4 weeks. From an initial number of 13 rats in each group, 6 were sacrificed for analysis. The remaining rats were continued on the same regimen as previously for 2 more weeks. During this time intakes of food and water were measured daily. Faeces from each group were collected and weighed daily during the 5th and 6th weeks, and analyses for moisture, nitrogen, fat and ash were done on half-weekly accumulations. At the end of 6 weeks the basal rats all exhibited severe xerophthalmia. Since previous work had shown that animals on this basal diet will not survive more than 7 weeks, the series was terminated at the end of the 6th week. Analyses of body composition were carried out on the 7 residual rats in each group.

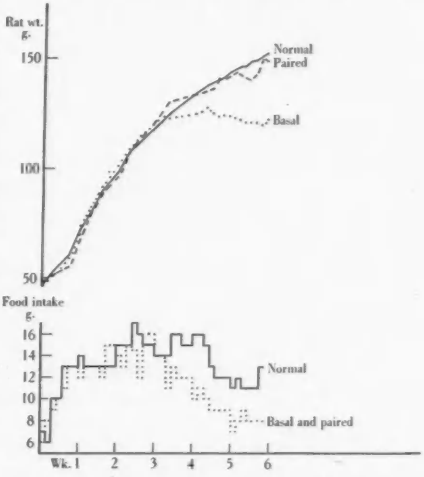


Fig. 1. Average food consumption and average weight curves for 3 groups of 7 rats each.

The bodies were prepared for analysis by freezing with CO_2 ice. The frozen bodies from each group were finely minced and then thoroughly mixed. Aliquots, for moisture determinations, were withdrawn from the mixed material and dried to constant weight in a vacuum oven at 50°. The dried aliquots were reground and mixed and fresh aliquots were used for determination of fat, N and ash. Fat was estimated by ether extraction in a Soxhlet apparatus, N by the Kjeldahl method (the factor 6·25 being used to convert N to protein) and ash was determined by ignition to constant weight in an electric furnace.

Results

Changes in body weight are shown in Fig. 1. Data on nitrogen and fat intake and excretion are given in Table 1. Tables 2 and 3 record results of analyses for the determination of body composition.

Table 1. *Intake and faecal excretion of N and fat (g./rat/day) for last 14 days*

	Normal	Basal	Paired
N intake	2·68	1·92	1·92
N in faeces	0·27	0·22	0·22
N absorbed	2·41 (90%)	1·70 (88%)	1·70 (88%)
Fat intake	0·15	0·11	0·11
Fat in faeces	0·04	0·03	0·05
Fat absorbed	0·11 (73%)	0·08 (73%)	0·06 (56%)

Table 2. *Average body composition of 6 animals from each group sacrificed at end of 4 weeks*

	Normal		Basal		Paired	
	%	g.	%	g.	%	g.
Water	61·1	94	66·0	87	63·6	91
Protein	17·8	27	19·7	26	18·4	26
Fat	18·5	28	9·9	13	13·8	20
Ash	2·6	4	3·0	4	3·0	4
Total	100·0	153	98·6	130	98·8	141
Body weight		154		132		143

Table 3. *Average body composition of 7 animals from each group sacrificed at end of 6 weeks*

	Normal		Basal		Paired	
	%	g.	%	g.	%	g.
Water	62·6	95	66·5	82	60·5	90
Protein	18·9	29	20·5	25	19·3	29
Fat	16·1	24	8·3	10	15·8	23
Ash	2·4	4	3·9	5	3·3	5
Total	100·0	152	99·2	122	98·9	147
Body weight		152		123		148

Discussion

Since the method of paired feeding was employed, any difference in the observed results between the basal and the paired group is due to the specific effect of vitamin A on metabolism or absorption.

Vitamin A was responsible for a 17% difference in weight between the basal and the paired groups. Results in agreement with this figure were obtained with the other 5 series, these being respectively 16, 20, 15, 20 and 25%.

The effect of food upon body weight is shown by the difference in weight between the paired group and the normal group. This effect varies considerably, depending upon the appetite of the animals in the basal group, from an insignificant value of 3% in this series to 20, 25, 8 and 23% in other series.

In the series reported 32% of the difference in body weight between the basal and paired groups was due to water. There was no appreciable difference between the water intake of the 3 groups and yet the basal group contained a higher percentage of water. This is in agreement with the results of Cameron [1938]. 52% of the weight difference between the basal and the paired groups was due to a favourable effect of vitamin A upon fat retention. The paired group absorbed less fat and yet had more fat in their bodies than the basal group. The remainder of the difference in weight (16%) between the paired and basal groups was due to a difference in the amount of protein.

The effect of vitamin A on body weight does not appear to be due to alterations in absorption of food but to utilization after absorption. According to Green [1934] fat can be absorbed normally in vitamin A deficiency.

Summary

Rats which were pair-fed and received vitamin A were significantly heavier than control animals deprived of the vitamin. The difference in weight was due to a loss of protein from the deficient animals and to a greater retention of water, fat and protein in the animals supplied with the vitamin. The difference was apparently not due to alterations in absorption of food.

The portion of this investigation carried out in the University of Toronto was made possible by a grant from the Division of Natural Sciences of the Rockefeller Foundation.

REFERENCES

Cameron [1938]. *J. Home Econ.* **30**, 585.
Coward [1938]. The biological standardization of the vitamins. London: Baillière, Tindall and Cox.
Green [1934]. *Biochem. J.* **28**, 25.
Sampson & Korenchevsky [1932]. *Biochem. J.* **26**, 1322.

92. The Relation between the Increase in Weight of Rats during the Dosing Period of Vitamin A and B_1 Determinations and the Standard Deviation of that Increase

By **Katharine Hope Coward**, *From the College of the Pharmaceutical Society, London*

(*Received 27 July 1942*)

Some years ago, in the early application of statistical methods to the analysis of results of vitamin determinations, Coward [1932] showed, by arranging 108 groups of rats used in vitamin A tests according to the average increase in weight of the groups, that the standard deviation of the increase in weight was independent of the size of the increase. The curve of response relating increase in weight to dose of vitamin A given was logarithmic in shape, and therefore the same degree of accuracy was obtainable whether the increase in weight was high or low. It has, however, lately seemed desirable to re-examine this point in view of possible improvements in the technique of carrying out the tests, and also to examine the results of vitamin B_1 determinations in the same way, for this has not been done before. The early result was fully confirmed and a similar result was obtained for the vitamin B_1 determinations.

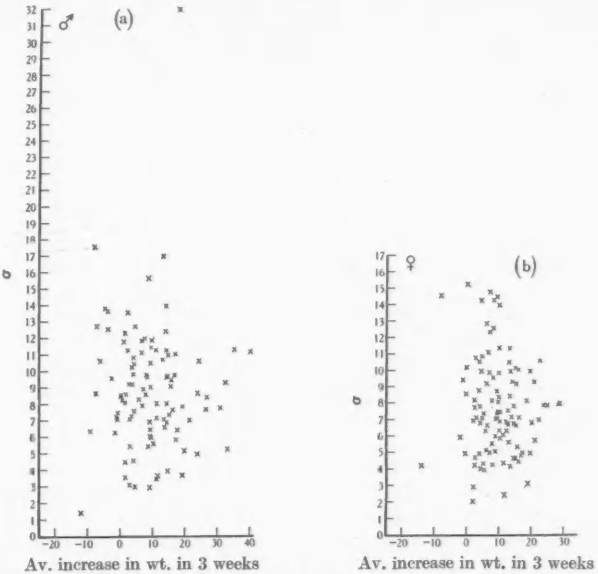


Fig. 1. Relation between the average increase in weight of rats during the dosing period of vitamin A determinations and the standard deviation of the increase, (*a*) male rats, (*b*) female rats.

Material for the calculations

Many hundreds of groups of rats used in vitamin A determinations in this laboratory have been available for assessing the standard deviation of the increase in weight during the dosing period. The last hundred or so of these groups, in fact all the groups used since the laboratory's evacuation from London, form the material from which conclusions are

drawn in this paper. Scatter diagrams relating increase in weight and standard deviation of the increase were made of the results from male and female rats respectively (Fig. 1 *a*, *b*) and it was so obvious that there was no correlation between these figures that no calculation of the correlation coefficient was made.

For the vitamin B_1 examination, the last 100 groups of rats used for vitamin B_1 determinations in this laboratory were chosen. The result may be seen in Fig. 2 *a*, *b*. It is obvious here also that there is no correlation between increase in weight during the dosing period and the standard deviation of the increase.

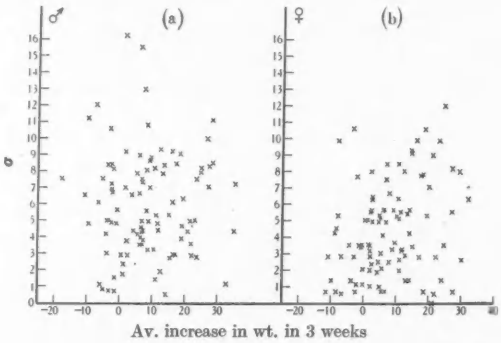


Fig. 2. Relation between the average increase in weight of rats during the dosing period of vitamin B_1 determinations and the standard deviation of the increase, (*a*) male rats, (*b*) female rats.

There appears to be some decrease in the average standard deviation of the increase in weight of rats used in vitamin A tests as compared with that previously reported by the writer [Coward, 1933]. In the last 100 or so groups the average standard deviation, $\sigma = \sqrt{\frac{\Sigma d^2}{N-M}}$ (in which d = the deviation from the mean of any group, N = the total number of rats and M = the number of groups), was found to be 9·58 for the male rats and 8·22 for the female rats, whereas the values found in the earlier work were 11·86 and 9·74 respectively.

There has been some decrease also in the standard deviation of the increase in weight of rats during a 3 weeks' test for vitamin B_1. In the results quoted in this paper the mean value of σ is 6·54 for male rats and 5·08 for female rats, whereas the values found in the earlier work [Coward, Burn, Ling & Morgan, 1933] were 8·16 and 7·42 respectively.

Summary

1. No correlation was found between the increase in weight of rats during the dosing period and the standard deviation of the increase, in either vitamin A or vitamin B_1 determinations.

2. Therefore as the curves of response relating increase in weight to dose of vitamin given are logarithmic, the same degree of accuracy is obtainable whether the increases in weight obtained in the determinations are high or low.

3. The standard deviation of the increases in weight of male and female rats in both vitamin A and vitamin B_1 determinations has decreased in the course of some years in this laboratory.

References

Coward, K. H. [1932]. *Biochem. J.* **26**, 691.
—— [1933]. *Biochem. J.* **27**, 445.
Coward, K. H., Burn, J. H., Ling, H. W. & Morgan, B. G. E. [1933]. *Biochem. J.* **27**, 1719.

93. The Estimation of Protein by the Biuret and Greenberg Methods

By **Frank Wokes** and **Bess M. Still**, *From the Biochemical Department, the College of the Pharmaceutical Society, Cardiff, and the Ovaltine Research Laboratories, King's Langley, Herts.*

(*Received 17 August 1942*)

For the estimation of small amounts of protein in biological fluids such as serum, urine and cerebrospinal fluid, as well as in certain foods such as milk, there have been many attempts to utilize colour tests. Perhaps the two most important of these are the biuret test, and the test for tyrosine introduced by Folin and his colleagues, and applied to serum, albumin and globulin by Greenberg [1929], who matched the colour against that produced by a standard tyrosine solution, factors being used to correct for the different tyrosine content of the two proteins. Fine [1935] applied the biuret test to these two serum proteins. The colour produced was matched in a colorimeter against that produced by a standard serum of which the protein content had been determined by Kjeldahl estimations. No correction factor was employed to distinguish between albumin and globulin, since equal weights of these were stated to produce equal intensities of colour. Lieben & Jesserer [1936] used the Zeiss-Pulfrich step-photometer to investigate the biuret test, and found that equal weights of casein, gelatin, egg white, fibrin, edestin and zein reacted with the same amount of copper. The biuret colour was said to be affected by the *p*H and by the concentration of Cu ions in the reaction mixture, the best results being given when a 3% NaOH and a 1% $CuSO_4$ solution reacted with 0·5% of protein. The biuret colour was stated to consist of two components, a red and a blue. The red component was stated to be characteristic of protein and was measured by means of a No. 47 filter, which showed an increase in this red component as the protein solution aged, but no figures were given for this increase. Sizer [1937], using a recording photoelectric spectrophotometer, determined the absorption band of the biuret colour given by casein, and found a maximum at about 520 mμ. Harrison [1937] introduced a simplified biuret test for estimating protein in serum, urine, cerebrospinal fluid and milk. The colour was matched against a graded series of coloured glasses in a Lovibond comparator. An equal weight of each protein was assumed to produce the same intensity of colour. Robinson, Price & Hogben [1937] returned to Fine's method of matching the biuret colour in a colorimeter against that given by a standard serum preserved with thymol, and introduced certain improvements in the technique. Robinson & Hogben [1940] repeated Sizer's spectrophotometric investigation of the biuret colour given by serum and obtained a similar band with a very blunt peak. They considered the maximum to be at 560 mμ, though their published curve showed no significant difference in density between 540 and 560 mμ. The biuret colour on standing 24 hr. changed from purplish red to red, but no difference was observed in the density values at 560 mμ up to 48 hr. Loss of protein occurred on filtration. Variation of the amount of $CuSO_4$ added from 1·25 to 2·5 ml. of a 20% solution per 100 ml. made no difference to the colour, but when 25 ml. of 2% solution was used the results became more variable and were influenced by the concentration of NaOH. The results obtained by their standard technique agreed with protein estimations by Kjeldahl over a wide range of samples.

Experimental

Tests on numerous clinical samples of urine and cerebrospinal fluid having shown that precipitation methods (e.g. Esbach, Aufrecht, Mestrezat) gave unreliable results, we turned our attention to colour tests. Greenberg's method was applied as described by Greenberg [1929]. The biuret test was employed as directed by Harrison [1937] with slight modifications mentioned below, where we also describe the calibration of the Lovibond glasses we used. This calibration showed that one of the chief sources of error in Harrison's method may be the inaccurate grading of Lovibond glasses, and special precautions were necessary to minimize the error. The other sources of error in both methods were minimized by adjusting the conditions until statistical analysis of the results showed that the percentage standard deviation had been made as small as possible. This required a large number of readings, especially when using the colorimeter. In fact, most of the results we quote are based each on some 50 or 60 readings taken independently by two different observers, thus ensuring an average percentage standard deviation ranging from 0·8 to 1·2 in our Greenberg results, so that a difference of 2 to 3% ($2\frac{1}{2}$ times the standard deviation) was considered significant. The biuret test by Harrison's method is much less accurate, the average percentage standard deviation being at least three times that for the Greenberg method, so that a percentage difference of less than 8 was not significant.

Estimation of total protein in serum

The total protein was estimated by the two colour tests in a number of dilutions of normal horse serum, of which the protein content had been determined by Kjeldahl determinations, using the protein/N factor of 6·55 proposed by Adair & Robinson [1930], after deducting the non-protein N which was determined separately. The results, some of which were obtained on a serum which was a few days old, are given in Table 1. They showed the following points.

1. The biuret method becomes unreliable when applied to dilutions below 1 in 50 (which frequently occur in clinical samples).

2. Omitting results below 1 in 50, the percentage standard deviation of the biuret method was 8·3, within the limits to be expected from statistical analysis of individual readings.

3. The percentage standard deviation for the Greenberg results was also within the expected range.

4. The mean result for the Greenberg estimations was significantly less than 100% of the true value. If, however, Adair and Robinson's factor of 6·55 was replaced by the usual protein factor of 6·25, the mean for the Greenberg results became 94% of the true value.

5. The mean result for the biuret method was significantly less than the true value.

Table 1. *Estimation of total protein in serum by biuret and Greenberg methods*

Results given as percentages of the true value as determined by Kjeldahl estimations.

Dilution of serum	Biuret	Greenberg
1 in 10	75	90
1 in 20	81	88
1 in 50	88	91
1 in 100	68	90
Mean	78	90
Standard deviation	6·5	1·4
% deviation	8·3	1·5

Table 2. *Estimation of total protein in different samples of normal serum by biuret and Greenberg methods*

Serum	Approx. age of serum (weeks)	Biuret	Greenberg
Horse II	1	109	78
Horse III	2	81	90
Human I	4	102	95
Human II	1	65	84
Mean		89	87
Standard deviation		20	7·4
% deviation		22·5	8·5

Estimation of total protein in different sera

The total protein of several samples of normal horse and human serum was estimated by the two colour tests. The results, again expressed as percentages of the true value as determined by Kjeldahl determinations, are given in Table 2. It will be seen that the

values obtained on fairly fresh sera were significantly lower than the true values, and that the estimations on older sera gave results which agreed with, or were greater than, the true values.

There was a definite increase in the values given by both methods as the serum aged.

Estimation of albumin and globulin in normal horse serum

The albumin and globulin were separated by Greenberg's sodium sulphate method, and the total protein was estimated in the two fractions by the biuret, Greenberg and Kjeldahl methods. Two separations (1st and 2nd) were made within a day or so of each other, then after an interval of several weeks two further separations (3rd and 4th) were again made within a day or so of each other. The serum throughout all our experiments was stored in a refrigerator and showed no signs of decomposition. The results of these experiments, again expressed as percentages of the true values, are given in Table 3, and from them the following facts emerge.

1. There was a definite increase in the values obtained on both protein fractions by both methods as the serum aged.
2. Results obtained within a day or so of one another agreed within the expected limits.
3. The biuret method gave higher results on albumin than on globulin, in parallel determinations.
4. The mean biuret result was significantly higher than the mean Greenberg result.

Some of the Greenberg results are omitted because the standard used was afterwards found to be unreliable.

Table 3. *Estimation of albumin and globulin in normal horse serum by biuret and Greenberg methods*

Results given as percentage of the true value as determined by Kjeldahl estimations.

	Approx. age of serum (weeks)	Albumin		Globulin	
		Biuret	Greenberg	Biuret	Greenberg
1st separation	4	97·8	79·7	92·2	—
2nd ,,	4	96·4	—	94·3	—
3rd ,,	8	122	88·2	109·2	82·7
4th ,,	8	127	89·1	113·8	84·5
Mean		110·8	85·7	102·4	83·6
Standard deviation		15·9	3·0	10·7	1·3
% deviation		14·3	3·5	10·5	1·5

Table 4. *Estimation of albumin and globulin in normal human serum by biuret and Greenberg methods*

Results given as percentages of the true value as determined by Kjeldahl estimation.

	Approx. age of serum (weeks)	Albumin		Globulin	
Citrated serum		Biuret	Greenberg	Biuret	Greenberg
1st separation	3	102	79·2	52·9	82·6
2nd ,,	6	114	83·2	83·8	88·5
3rd ,,	6–7	105	81·0	70·8	89·7
Mean		107	81·1	69·2	86·9
Standard deviation		6·2	2·0	18·1	3·8
% deviation		5·8	2·5	26·2	4·4
Oxalated serum					
1st separation		90·4	83·4	76·2	90·3

Estimation of albumin and globulin in normal human sera

Similar experiments were carried out on normal human sera. The first sample used was obtained from citrated blood collected for transfusion purposes and stored aseptically in the refrigerator for 3 weeks before we examined it. The first separation of the proteins

in this sample was made when it was 3 weeks old, the second when it was nearly 6 weeks old and the third when it was between 6 and 7 weeks old. The second sample of human serum was obtained from freshly collected oxalated blood, separated within an hour of collection and examined a few days later. The results, given in Table 4, on the whole confirmed those already obtained on horse serum in showing that as the serum aged the results by both methods became higher, and that the biuret method gave higher results on albumin than on globulin.

Precautions taken with the tyrosine standard

The results we had obtained by Greenberg's method might be attributed to our tyrosine standard being (*a*) weak, or (*b*) unstable. Precautions were taken to avoid both of these possibilities. Tests showed that the sample of tyrosine used as standard was of satisfactory purity, and that the solutions of this tyrosine made freshly every week or two and stored in the refrigerator showed no deterioration when checked against new solutions. Moreover, when discussing this point with Dr Rimington, who had also been using tyrosine as a standard in the Greenberg method, we found that he also had obtained low results similar to ours. We therefore concluded that the low results were due to a gradual change in the serum, and tested this conclusion by direct experiment.

Effect of 'age' of serum on Greenberg results

A sample of normal oxalated blood was centrifuged immediately after collection to separate the serum, and the total protein was then estimated in the serum by Greenberg's method. The time from the removal of the blood from the vein to the point when half the readings had been taken on the Klett colorimeter was approximately 60 min., which was considered to be the 'age' of the serum when the first estimation was made. The estimation was repeated at intervals until at the end of 40 days the change in the serum, which was stored in the refrigerator, had practically ceased. The results by the Greenberg method were then 88% of the true values (Table 5). Out of 21 results obtained by the

Table 5. *Effect of age of serum on results given by Greenberg's method for total protein*

Results given as percentages of the true value as determined by Kjeldahl estimations.

Age of serum	Greenberg result	Age of serum	Greenberg result
1 hr.	67	23 hr.	81
2 ,,	74	3 days	83
3 ,,	75	4 ,,	87
4½ ,,	79	40 ,,	88
22 ,,	80		

Note. The 'age' of the serum was calculated from the time when the blood was removed from the vein to the time when half the readings had been taken on the Klett colorimeter, on the assumption that changes might still be taking place in the protein after it was precipitated. It might be more accurate to calculate the age up to the time when the protein was precipitated. This would reduce each of the 'ages' in the above table by about 30 min.

Greenberg method on different samples kept for times ranging from a few days to several months and recorded in Tables 1–4, only one was significantly higher than 88%. Thus it appears that the Greenberg method usually gives results lower than the true value, but it would not be safe to assume that this will always occur, since certain factors such as storage conditions might cause the change in the serum to proceed further. Moreover, if the usual protein/N factor of 6·25 is employed instead of Adair and Robinson's factor of 6·55, the highest results obtainable by Greenberg's method will be at least 5% higher than those recorded above.

Effect of age of serum on biuret results

This effect was more difficult to investigate because of the greater experimental error of the biuret method as employed by Harrison. Statistical analysis of the data from a large number of biuret estimations showed that a difference of less than 6 or 7% could not be considered significant, and after calibrating the Lovibond glasses we had been using we realized that the experimental error might be much greater. This led us to try the procedure of Fine [1935] and of Robinson & Hogden [1940], of matching the biuret colour in a Klett colorimeter against the colour produced by a standard serum. For this purpose we used a serum several weeks old in which, by analogy with the Greenberg results, the change might be expected to have practically ceased, or at any rate to be progressing so slowly that results obtained within a few hours of each other using it as a standard would be comparable. We found this gave much greater accuracy than the Lovibond glasses, but the experimental error was still more than twice that of the Greenberg method, largely because of the difficulty of matching the blend of two colours. We also introduced the following modifications of Harrison's technique: (1) a 1 in 10 dilution of the serum was employed, of which 2 ml. could be measured more accurately than 0·2 ml. of undiluted serum; (2) after precipitating the protein with trichloroacetic acid and centrifuging, the supernatant fluid was passed through glass wool in a small funnel to collect any particles of protein inadvertently brought over. These particles were washed with distilled water, and the funnel was then placed on the centrifuge tube containing the bulk of the precipitated protein; 1 ml. of 30% NaOH was dropped on to the funnel to dissolve and carry through any protein particles on the glass wool which was then rinsed with 2 or 3 ml. distilled water. 1 ml. 5% $CuSO_4$ was added to the contents of the centrifuge tube, which were then made up to 10 ml., the calibration of the tubes used being carefully checked. The contents were mixed by shaking and centrifuged; the clear supernatant fluid was then drawn off for colorimetric estimation.

The serum was separated from a sample of oxalated blood immediately after withdrawal from the vein, and the total protein in the serum was determined by the biuret method as rapidly as possible, and afterwards at frequent intervals until the first rapid change in the serum appeared to be slowing down. The results, expressed as percentages of the true value as shown by Kjeldahl estimations, are given in Table 6. It will be seen

Table 6. *Effect of age of serum on results given by biuret method for total protein*

Results given as percentage of true value as determined by Kjeldahl estimations.

Time after collection of blood	Biuret result by Tintometer [Harrison]	Biuret result by Klett colorimeter [Robinson & Hogden]
1 hr.	51	—
2 „	73	71
3 „	91	—
4 „	90	80
5 „	88	—
24 „	86	81
3 days	86	—

that with the biuret method, as with Greenberg's method, the values gradually increase as the serum ages. Because of the greater experimental error the evidence was less convincing than with the Greenberg method, and we therefore decided to apply spectroscopic methods in the hope of obtaining more conclusive results.

Spectrophotometric determination of the biuret absorption band

Sizer [1937], using a recording photoelectric spectrophotometer, had determined the absorption band of the biuret colour given by casein, and found a maximum at about 520 mμ. Robinson & Hogden [1940], using a Bausch and Lomb universal spectrophotometer, stated that the maximum was at 560 mμ. A number of workers have observed with the naked eye changes in the biuret colour on standing which might be expected to affect the spectrum. We therefore decided to determine the absorption band of biuret colours given by casein and by serum proteins in solutions of different ages. For this purpose we employed a Hilger-Nutting constant deviation wave-length spectrophotometer, which we calibrated for wave-length measurements against the emission spectrum of a helium lamp, and for density measurements against a set of neutral glasses standardized by the National Physical Laboratory. The solution of the biuret colour, carefully freed from all particles, was placed in a 20 mm. cell of Hilger's type D, whilst a precisely similar cell containing the blank (biuret test carried out on distilled water) was placed in the other beam of light.

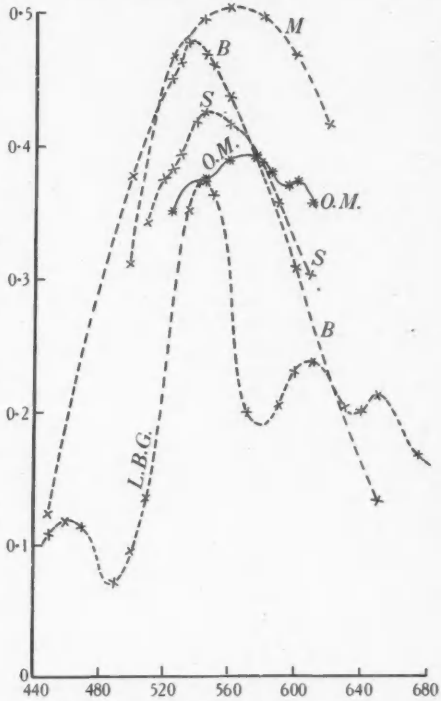


Fig. 1. Absorption spectra of biuret colour with proteins of fresh milk (*M*), of milk after 7 days' storage in refrigerator (*O.M.*), and of fresh serum (*S*), also of biuret itself with $CuSO_4$ and NaOH (*B*) and of 180 Lovibond biuret glass (*L.B.G.*) from the set used for protein estimations. Ordinates represent mean densities; abscissae represent wave-lengths.

Measurements of the density at each wave-length were made independently by two observers, each of whom took 2 pairs of readings with each eye with the biuret colour in the lower beam of light and the blank in the upper beam, followed by another 2 pairs of readings with each eye with the positions of the biuret colour and blank reversed. The first reading in each pair was taken with the lower colour just perceptibly darker than the upper colour, and the second reading with the upper colour just perceptibly darker. This procedure was found to be more satisfactory than attempting to obtain exact matches of colour. The average percentage standard deviation for 4 pairs of readings was brought by careful working below 2, corresponding to a standard deviation of less than 1% for the 16 pairs of readings on which the final figure for the density readings at each wave-length was based. Hence a difference of $2\frac{1}{2}$% (i.e. $2\frac{1}{2}$ times the standard deviation) was considered significant.

Numerous experiments were carried out under different conditions, some of the results being summarized in Fig. 1. The following conclusions were reached.

(1) The absorption maxima for the biuret colour are approximately: on serum proteins 545–555 mμ, on fresh milk proteins 555–565 mμ, and for biuret itself 535 mμ.

(2) As a casein solution ages the absorption maximum of its biuret colour shifts slightly towards longer wave-lengths (e.g. from 560 to 570 mμ after 7 days at 0°).

(3) With solutions of a given protein of the same age the density readings at the maximum are proportional to the concentration of the protein.

The precise maxima for both serum and milk proteins are difficult to determine spectrophotometrically, because of the changes in density and the shift of the peak of the absorption band which are taking place whilst the determination is being made. If a recording photoelectric spectrophotometer had been available, permitting determination to be completed within a short time after separation of the serum, it is possible that the initial maximum would have been found at 520 mμ as Sizer reports. We therefore decided in our quantitative experiments on serum described below to determine the density at 525 mμ, which is probably quite close to the maximum for the biuret colour on freshly separated serum.

Spectrometric study of the effect of ageing of serum proteins on their biuret colour

The spectrophotometer was next employed to study the changes taking place in serum proteins after their removal from the body. The serum was separated from a sample of normal citrated human blood by centrifuging immediately after it had been withdrawn from the vein, and was quickly divided into 3 portions, one of which was placed in the refrigerator, the second in an incubator at 37° and the third was kept at room temperature. The biuret test was at once carried out on a sample of the serum kept at room temperature. The clear supernatant fluid from this test was put in the type D cell and the density determined at 525 mμ by the procedure described above. The first observer started taking readings with the biuret colour in the lower beam, whilst the second observer immediately afterwards started taking readings with the biuret colour in the upper beam. Thus, even if the intensity of the colour were changing during the estimation, the mean of all the readings could be taken as the true value at the time when half the readings had been taken. This was an hour after withdrawal of the blood from the vein, and half an hour after addition of the trichloroacetic acid.

The blood pipettes used to measure the serum had been calibrated with Hg, and corrections were made correspondingly. The calibrations of the centrifuge tubes were also checked.

The estimation was repeated at intervals on the 3 portions of serum, duplicates being run to test the accuracy of the method. The results are recorded in Table 7.

Table 7. *Effect of age of serum and its storage temperature on results given by biuret test using spectrophotometer*

Results given as average densities at 525 mμ.

Age of serum (hr.)	Storage temperature		
	5°	20–25°	37°
1	—	0·641 ±0·008	—
1½	—	0·684 ±0·004	—
2	—	—	0·715 ±0·004
5	0·627 ±0·013	—	0·614 ±0·005
7	0·657 ±0·032	—	—
24	0·672	0·755 ±0·005	—

(1) There was a steady increase in the density readings in the region of maximum absorption, which confirmed the previous findings that the biuret test, like the Greenberg test, gives higher results with older sera.

(2) The rate of this increase depended on the temperature at which the serum was stored.

(3) The mean percentage standard deviation for 7 pairs of duplicate estimations was 2·44, rather higher than was expected from the standard deviation of individual readings.

A difference of 6% was therefore considered significant. The actual increases in density recorded were 11·5% in 2 hr. at 37°, 17·7% in 24 hr. at room temperature, and 7·2% in 24 hr. at 5°. All of these were significant.

(4) The rate of increase in the colour intensity at room temperature from 1 to 1½ hr. was about 7% per hour, much less than had been observed with the tintometer. This was due probably to the much higher experimental error when using the tintometer. The increase observed spectrophotometrically from 1½ to 24 hr. at room temperature was 10·4% as compared with 14% from 2 to 24 hr. in the previous experiment using the Klett colorimeter.

Spectrophotometric study of the effect of ageing of milk proteins on their biuret colour

A sample was taken of the morning milk from Pacified Princess, one of the herd of pedigree Jersey cows on the Ovaltine Dairy Farm. The milk was at once divided into 3 portions, one of which was placed in the refrigerator, the second in the incubator at 37° and the third kept at room temperature. Within 30 min. of the commencement of milking the protein was precipitated with trichloroacetic acid in the usual manner, and the biuret colour was produced and its density at 560 mμ determined in the spectrophotometer. These determinations were repeated at intervals throughout the day on freshly precipitated protein from the 3 samples kept at different temperatures, readings being made in the manner described above, and duplicate precipitations and determinations usually being made. The results, recorded in Table 8, showed the following changes in the intensity of the colour as measured by the density at 560 mμ: (1) a steady increase such as had previously been observed with serum proteins; this was more rapid at higher temperatures; (2) a decrease on storage in the incubator for a prolonged period.

Table 8. *Effect of age of milk and its storage temperature on results given by biuret test using spectrophotometer*

Results given as average densities at 560 mμ.

Age of milk	Storage temperature 5°	15–20°	37°
1 hr.	—	0·540	—
2 ,,	—	—	0·646 ±0·005
3 ,,	—	0·568 ±0·005	—
4½ ,,	0·543 ±0·003	—	—
5½ ,,	—	—	0·816 ±0·019
7½ ,,	0·547	—	—
24 ,,	0·553	0·590 ±0·001	0·661 ±0·034
48 ,,	0·571 ±0·002	—	—
6 days	0·693 ±0·002	—	—

The increase in colour intensity was clearly seen in all 3 portions of the milk, and was in accordance with all our previous observations on serum. The final decrease in the incubator sample had previously been observed with serum kept in the incubator, and probably represented early stages in the decomposition of the protein.

The reliability of the results is shown by the deviations from the mean in 8 pairs of duplicate estimations which are given in Table 8, and by the percentage standard deviations, which ranged from 0·29 to 7·71 and averaged 2·14. The deviations were largest in the incubator samples, possibly due to the greater instability of the protein at higher temperatures, but even here the differences between the 2 and 5½ hr. samples, and between the 5½ and 24 hr. samples, were significant. If the results on the decomposing 24 hr. incubator sample are omitted, the average percentage standard deviation on the remainder of the samples is 1·4, for which a difference of 3–4% would be considered significant. In our opinion this figure gives a fair indication of the accuracy obtainable with the biuret method as we have employed it spectrophotometrically. Fine [1935]

considered that the error of the biuret method using a colorimeter should rarely exceed 5%. He did not, however, take into account any effect due to ageing of the serum. If the tintometer is used in the biuret method as described by Harrison the error may exceed 16%.

In our experiments on the biuret method applied to milk, readings taken at different wave-lengths at different times indicated a slight shift in the peak of the absorption band of the biuret colour as the milk aged, but our results showed that this shift played no significant part in the changes described above.

Spectrophotometer examination of the Lovibond biuret glasses

The deviations we observed in duplicate biuret estimations using Lovibond glasses were much wider than those found by Fine using a colorimeter and by ourselves using either the Klett colorimeter or the spectrophotometer. Suspecting that this might be due to inaccuracies in the Lovibond glasses, we undertook a spectrophotometric examination of the glasses with two objects: (1) to discover whether the colour of the glasses was a fair match for the biuret colour produced by serum or milk proteins, (2) to determine the degree of accuracy with which the various glasses had been graded.

All the determinations were made on a set of glasses, ranging from 20 to 180 on the scale, which was used throughout our experiments, and had also been employed in the College by several hundred students and post graduate workers for determining serum and milk proteins by Harrison's method.

Each glass in turn was carefully cleaned and polished, and placed first in the lower beam, at right angles to it, where two pairs of readings were taken with each eye, by two independent observers, in the manner described above. The glass was then transferred to the upper beam, and the same number of readings taken. Thus the final figure for the density at each wave-length was based on a total of 32 readings, giving results in which a difference of 2–3% was considered significant, the error being greater at certain wave-lengths than at others. The results are plotted in Fig. 1, the curve showing a main peak at about 545 mμ falling within the range of the peaks of serum and milk protein biuret colours, but complicated by the presence of subsidiary peaks at about 460, 605 and 650 mμ which have no counterpart in the true biuret colour. Any error produced by these subsidiary peaks could, however, hardly account for the wide deviations observed in duplicate biuret estimations by the method of Harrison using Lovibond glasses.

Calibration of the Lovibond biuret glasses

Density determinations were made as described above at the peak at 540–545 mμ in each of the glasses graded from 20 to 180. If the glasses had been correctly graded the ratio of the density to the grading of each glass should have been fairly constant. The results

Table 9. *Calibration of Lovibond biuret glasses*

Grading of glasses	Mean density at 540–545 mμ	Grading / Density	Deviation of grading density from mean for all glasses	% deviation from mean
180	0·374	481	+ 82	20·6
160	0·376	425	+ 26	6·5
140	0·383	365	− 34	8·5
120	0·406	296	−103	25·8
100	0·338	296	−103	25·8
80	0·262	305	− 94	23·6
60	0·189	317	− 82	20·6
40	0·064	625	+226	56·6
20	0·042	481	+ 82	20·6
Mean for all glasses		399	—	23·2
Mean omitting result on 40 and 20 glasses		355	—	16·6

recorded in Table 9 show that the grading of the glasses was unsatisfactory, the deviation from the average density/grading ratio ranging from 6·5 to 56·6%, and averaging 23·2%. In actual practice the 20 and 40 glasses were rarely used, and the average error of 16·6% in the grading of the remainder gives a fairer indication of the error incurred when using this set of glasses. This error is considerably higher than the errors previously observed in the calibration of other Lovibond glasses [cf. Gibson & Harris, 1927; Wokes, 1937], and it must not be assumed to be typical of the general run of Lovibond biuret glasses; it is clear, however, that more calibrations of these glasses are desirable.

Summary

The results given by the biuret and Greenberg methods of estimating protein in serum examined within an hour or two of removal of the blood from the body are lower than the values given by determinations of protein N by Kjeldahl. This applies both to total protein and to albumin or globulin separated from horse or human sera, oxalated or citrated. As the serum ages the results become higher, the rate of increase being more rapid at higher temperatures.

Spectrophotometric determination of the absorption curve of the biuret colour gave the following maxima: fresh serum protein 545–555 mμ, fresh milk protein 555–565 mμ, biuret itself 535 mμ. As casein solutions age the biuret maximum shifts slightly towards longer wave-lengths at about 570 mμ, and indications appear of the presence of two subsidiary peaks at about 540 and 600 mμ.

Density determinations with the spectrophotometer in the region of maximum absorption confirmed the findings obtained with the tintometer and Klett colorimeter that the results increase with ageing of serum, and extended the findings to milk.

Spectrophotometric examination of a set of Lovibond biuret glasses used for protein estimations by Harrison's method showed a main peak at about 545 mμ, also subsidiary peaks at about 460, 605 and 650 mμ which do not appear in the biuret colour on fresh protein and may therefore lead to inaccurate results. A more serious error in this method is inaccurate grading of the glasses, which, judged by the results obtained on the set examined, may lead to errors exceeding 16%.

We are indebted to Prof. A. M. Kennedy, Director of the Medical Unit at the Royal Infirmary, Cardiff, for kindly enabling us to obtain fresh samples of blood and serum, to Mr Davis, Manager of the Ovaltine Dairy Farm, for facilities in obtaining samples of milk, and to Miss Joan Organ for assistance in calibrating the Lovibond glasses on the spectrophotometer.

REFERENCES

Adair, G. S. & Robinson, M. E. [1930]. *Biochem. J.* **24**, 993, 1864.
Fine, J. [1935]. *Biochem. J.* **29**, 799.
Gibson, K. S. & Harris, F. K. [1927]. *U.S. Bureau of Standards Sci. Papers*, No. 547.
Greenberg, D. M. [1929]. *J. biol. Chem.* **82**, 545.
Harrison, G. A. [1937]. *Chemical Methods in Clinical Medicine.* London: Churchill.
Lieben, F. & Jesserer, H. [1936]. *Biochem. Z.* **285**, 36.
Robinson, H. W. & Hogden, C. G. [1940]. *J. biol. Chem.* **135**, 707.
Robinson, H. W., Price, J. W. & Hogden, C. G. [1937]. *J. biol. Chem.* **120**, 481.
Sizer, I. W. [1937]. *Proc. Soc. exp. Biol., N.Y.*, **37**, 107.
Wokes, F. [1937]. *A Textbook of Applied Biochemistry*, p. 130. London.

94. A General Method for the Estimation of α-Keto-acids, and its Application to α-Keto-acid Metabolism in Pigeon Brain

By **Cyril Long**, *From the Department of Biochemistry, The Queen's University, Belfast*

(*Received 23 July 1942*)

The acceptance of the central position occupied by pyruvic acid in the intermediary metabolism of carbohydrate in animal tissues has recently led to a number of investigations on the behaviour of other α-keto-acids towards those enzymes which act upon pyruvic acid. The next higher homologues of pyruvic acid, namely, α-ketobutyric and α-ketovaleric acids, have received special attention [Green, Needham & Dewan, 1937; McGowan & Peters, 1937; Long & Peters, 1939; Cohen, 1939].

It has generally been observed that the effect produced by an enzyme on an α-keto-acid decreases progressively as the C-chain in the molecule is lengthened, i.e. the higher members of the series are less readily attacked than the lower members. An apparent exception, however, is the finding by Long & Peters [1939] that pyruvic and α-keto-butyric acids were equally rapidly utilized by a minced preparation of pigeon brain. Since there was not available, at that time, a satisfactory method of estimating small amounts of α-ketobutyric acid, their evidence was necessarily somewhat indirect. More recently, however, it has been felt that this claim should be re-examined by means of direct observations, and since it was intended to continue the investigation of α-keto-acid metabolism, both in brain and in other tissues, it was clearly desirable to develop a method for the accurate estimation of small amounts of α-keto-acids of the order 0·1–1·0 μmol.

For this purpose, the bisulphite-binding method of Clift & Cook [1932] was tested but proved to be unsatisfactory on account of the low recoveries obtained, even when using the pure Na salts of the α-keto-acids. The method finally adopted was based on that used by Lu [1939] for the determination of pyruvic acid in blood filtrates. By this means it was possible, with a high degree of accuracy, to estimate all the α-keto-acids studied. When the method was applied to the problem of the comparative utilization of pyruvic and α-ketobutyric acids in minced pigeon brain, the findings of Long & Peters [1939] were confirmed in detail.

Experimental

(1) *Reagents*

Na pyruvate. A gift from Prof. R. A. Peters; prepared from pure pyruvic acid [Peters, 1938].

Na α-ketobutyrate. Prepared according to Long & Peters [1939].

Na α-ketovalerate. Specimen used by Long & Peters [1939].

Na glyoxylate. Glyoxylic acid (1 g. B.D.H.) in conc. aqueous solution was carefully adjusted with NaOH (40%) to *p*H 6, under ice-cold conditions. Addition of acetone caused precipitation of the Na salt. Recrystallized three times from aqueous acetone.

α-Ketoglutaric acid. Prepared according to Neuberg & Ringer [1915]. Since the free acid was a stable solid, it was not necessary to convert it into its Na salt.

(2) *The purity of the Na salts of the α-keto-acids*

Krebs [1938] showed that standard solutions of pure α-ketoglutaric acid, when treated with excess of acid $KMnO_4$ at room temperature, yielded the theoretical amount of CO_2 for oxidative decarboxylation, an equivalent of succinic acid being simultaneously produced. It is clear that the oxidative decarboxylation is dependent on the presence of the —CO.COOH group only, and must be given by all α-keto-acids.

$$R.CO.COOH + O \rightarrow R.COOH + CO_2.$$

The reaction has been used to determine the purity of the Na salts of those α-keto-acids listed above.

A solution containing 10–15μmol. of the Na salt of the α-keto-acid, dissolved in 5% H_2SO_4 (total vol. 2 ml.), was placed in the main chamber of a Dixon-Barcroft flask. $KMnO_4$ (approx. *N*; 0·2 ml.), contained in a Keilin cup, was added after temperature equilibration. The oxidation was conducted at 28° in air. CO_2 production was in all cases complete after 5 min. The CO_2 evolved in a control experiment in which 5% H_2SO_4 (2 ml.) was similarly treated, was deducted from the observed CO_2 production. This correction generally amounted to about 10μl. (about 3% of the total).

In Table 1 are shown the results obtained for the oxidation of the four Na salts of the α-keto-acids and for free α-ketoglutaric acid.

Table 1. *Evolution of CO_2 from α-keto-acids by acid $KMnO_4$, as an index of purity*

Each experiment represents the average of triplicate CO_2 productions, agreeing to ±1%. Recoveries calculated to nearest 0·5%.

	Exp.	Net CO_2 μl.	α-Keto-acid (μmol.) Calc.	α-Keto-acid (μmol.) Taken	% recovery
Na pyruvate	1	317	14·2	14·3	99·5
	2	378	16·9	16·8	100·5
	3	354	15·8	15·8	100
	4	392	17·5	17·5	100
	5	343	15·4	15·4	100
Na α-ketobutyrate	6	325	14·4	14·5	99·5
	7	329	14·7	14·5	101·5
	8	307	13·7	14·0	98
	9	304	13·6	13·6	100
Na α-ketovalerate	10	312	13·9	14·2	98
	11	351	15·7	15·7	100
	12	304	13·6	13·4	101·5
α-Ketoglutaric acid	13	334	14·9	14·7	101·5
	14	353	15·7	15·5	101·5
	15	380	17·0	16·9	100·5
Na glyoxylate	16	174	7·8	7·7	101
	17	368	16·4	16·3	100·5

From the values given in Table 1, it is seen that, within experimental error, all the α-keto-acids studied yielded the theoretical CO_2 production on treatment with acid $KMnO_4$. The samples must therefore be considered quite pure.

In the cases of α-ketoglutaric acid and the Na salts of pyruvic, α-ketobutyric and α-ketovaleric acids, the CO_2 production indicated in Table 1 is the amount actually observed, less the control. With Na glyoxylate, however, the evidence for absolute purity is not so direct, since formate which is produced by oxidative decarboxylation is itself slowly oxidized, with simultaneous CO_2 production, under the given conditions. Fig. 1 shows the extent of this oxidation at different time intervals. The graph connecting these two variables is a smooth curve, which on extrapolation to zero time gives the CO_2 evolution independent of formate oxidation. The extrapolated values are those quoted in Table 1.

It was unfortunate that this method could not be applied directly to the estimation of α-keto-acids in biological fluids, its two main disadvantages being lack of specificity and the fact that a relatively large amount of the α-keto-acid would be required. Thus in order to obtain a reasonably accurate estimation by this method, about 5 μmol. of the α-keto-acid would be needed, whereas in the protein-free filtrates from micro-metabolic processes it would be necessary to estimate 0·1μmol. with the same degree of accuracy.

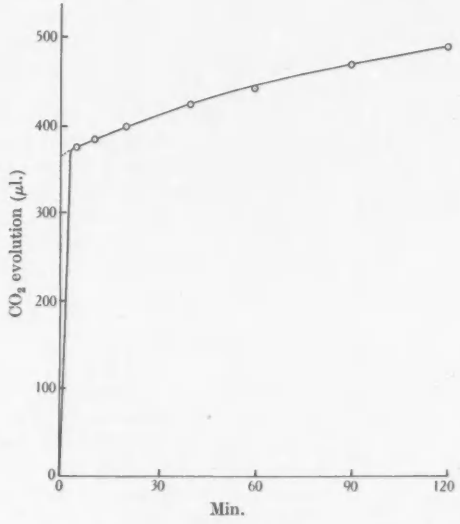


Fig. 1. The time course of CO_2 evolution from Na glyoxylate on treatment with acid $KMnO_4$ at 28°. Extrapolation to zero time is indicated by the discontinuous portion of the curve. Exp. 17.

(3) *The recovery of α-keto-acids by the bisulphite-binding method*

According to Clift & Cook [1932], standard solutions of pyruvic acid can be estimated by the bisulphite-binding technique with recoveries varying between 97 and 103 % (average value 100 %). Using freshly prepared solutions of Na pyruvate, with solid Na_2HPO_4 to liberate bound bisulphite [Lehnartz, 1928], it has not been possible in this work to obtain such a quantitative recovery, the average value being about 96·5 %. With the Na salts of other α-keto-acids and with free α-ketoglutaric acid, the recoveries have been even lower (Table 2).

Both first and second end-points of the titration with iodine were perfectly sharp, except in the case of α-ketoglutaric acid. Here, although the second end-point at alkaline reaction was quite definite, the first end-point at acid reaction showed considerable fading of colour. This variability in the case of α-ketoglutaric acid probably explains the lack of reproducibility observed in Exps. 15–18 (Table 2).

In view of the low recoveries obtained in Table 2, it was decided that the bisulphite-binding technique was unsatisfactory for accurate work and another method had to be sought. It may be noted that a further disadvantage of the method is its limited application to studies of α-keto-acid metabolism since it cannot be used in the presence of arsenite, which is widely used as a specific inhibitor of α-keto-acid oxidations.

Table 2. *The recovery of α-keto-acids by the bisulphite-binding method*

Each experiment represents the average of closely agreeing triplicate titrations, except in the case of α-ketoglutaric acid (see text). Recoveries calculated to the nearest 0·5 %.

	Exp.	*N*/100 iodine ml.	α-Keto-acid (μmol.) Calc.	Taken	% recovery
Na pyruvate	1	1·776	8·88	9·18	96·5
	2	1·669	8·35	8·85	94·5
	3	0·908	4·54	4·68	97
	4	0·896	4·48	4·59	97·5
	5	0·905	4·53	4·77	95
	6	1·095	5·48	5·59	98
	7	1·003	5·02	5·27	95·5
				Average	96·5
Na α-ketobutyrate	8	0·814	4·07	4·72	86
	9	0·839	4·20	4·84	87
	10	0·804	4·02	4·64	86·5
	11	0·766	3·83	4·52	84·5
	12	0·779	3·90	4·60	85
				Average	86
Na α-ketovalerate	13	0·838	4·19	4·73	88·5
	14	0·920	4·60	5·22	88
				Average	88
α-Ketoglutaric acid	15	1·380	6·90	7·77	89
	16	0·860	4·30	5·63	76·5
	17	1·033	5·17	6·49	79·5
	18	0·946	4·73	5·67	82
				Average	82
Na glyoxylate	19	0·745	3·73	4·63	80·5
	20	1·328	6·64	8·13	81·5
				Average	81

(4) *General colorimetric method for the estimation of α-keto-acids*

Recently, Lu [1939] has applied the condensation in acid solution between 2:4-dinitrophenylhydrazine and pyruvic acid to the determination of the latter in blood filtrates by means of the intensity of the red-brown colour developed when the pyruvic acid 2:4-dinitrophenylhydrazone so formed is treated with excess NaOH. With certain modifications, this method has been used to estimate those α-keto-acids listed above. It is especially suitable for the accurate determination of 0·1–1·0 μmol. of the α-keto-acid.

A standard curve connecting concentration and colour intensity for each α-keto-acid was obtained as follows. Known amounts of the Na salt, viz. 0·9, 0·6, 0·3 and 0·15 μmol., dissolved in 5 % trichloroacetic acid (total vol. 2 ml.) together with a control consisting of 5 % trichloroacetic acid (2 ml.) were measured into five test-tubes. The following details apply to each of the five samples. The acid solution was treated with 0·2 % 2:4-dinitrophenylhydrazine hydrochloride in 2*N* HCl (1 ml.). After 15 min., during which all the α-keto-acid was converted into its 2:4-dinitrophenylhydrazone, ethyl acetate (2 ml.) was added, and the aqueous solution extracted using a teat-pipette. For this purpose, the liquid in the tube was rapidly drawn into and expelled from the teat-pipette 15 times, an operation requiring about 20 sec. The lower aqueous layer was transferred to another test-tube and extracted twice more with ethyl acetate (1 ml. each time). The three ethyl acetate extracts were combined and the aqueous layer discarded. This solution in ethyl acetate of the 2:4-dinitrophenylhydrazone together with unchanged 2:4-dinitrophenylhydrazine was then extracted with three successive 2 ml. portions of 10 % Na_2CO_3, and the combined Na_2CO_3 extracts washed once with ethyl acetate (1 ml.). After allowing at least 50 min. for the Na_2CO_3 extract to clear, this pale yellow solution

was quantitatively transferred to a 10 ml. measuring cylinder, treated with 3 ml. 2*N* NaOH, made up to 10 ml. with distilled water and thoroughly mixed. This treatment with alkali caused a deep red-brown colour to develop. The intensities of absorption of the five samples were compared in a Spekker Absorptiometer, using a purple colour filter. (The Hilger No. 7 filter from the H.455 set, which was used in the present investigation, absorbed completely between 520 and 680 mμ and transmitted maximally between 400 and 360 mμ.) The drum reading with the control was taken as the zero. In this manner the curves in Fig. 2 were obtained.

In order that the method described may be used with the greatest accuracy and reliability, a number of experimental details need stressing. First, it is essential that the same reagent specimens be used for determining the standard curve and for the estimation of unknown quantities of the given α-keto-acid. It has been found that the ethyl acetate specimen, especially, must not be changed. It is equally important that the 0·2% solution of 2:4-dinitrophenylhydrazine hydrochloride in 2*N* HCl should be fairly fresh and must remain clear. The curves shown in Fig. 2 are those obtained using a given set of reagents. With other reagents, and especially when different samples of ethyl acetate have been employed, the colour intensity has varied quite considerably (±20%). However, when the reagents are unchanged, the same standard curve is always obtained. This is shown in the case of pyruvic and α-ketobutyric acids in curves *A* and *D*, Fig. 2. Another point of practical importance is that sufficient time (15 min.) should be allowed for the α-keto-acid to react with the 2:4-dinitrophenylhydrazine in acid solution, before the first extraction with ethyl acetate. With shorter time intervals, the reaction is incomplete, and low values are obtained when the absorption intensity is ultimately determined. Finally, a sufficient quantity of alkali must be used for the colour development. Lu [1939] recommended the use of 4 ml. *N* NaOH. When this amount was used, it was found that the absorption intensity diminished quite rapidly during the determination. However, when the quantity was increased to 3 ml. 2*N* NaOH, the intensity became stabilized over at least 90 min. [cf. also Bueding & Wortis, 1940].

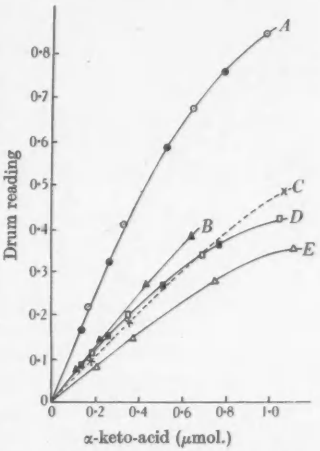


Fig. 2. The absorption intensity (drum reading) of the 2:4-dinitrophenylhydrazones of α-keto-acids in excess NaOH as a function of concentration. Purple filter, No. 7 Hilger (H.455). Curve *A*, pyruvic acid; curve *B*, α-ketovaleric acid; curve *C*, glyoxylic acid; curve *D*, α-ketobutyric acid; curve *E*, α-ketoglutaric acid. Curves *A* and *D* each show two experiments.

From the curves in Fig. 2 it will be seen that at any given molecular concentration, the colour complex from pyruvic acid possesses a greater intensity of absorption than that from any other member of the series. Its next higher homologue, α-ketobutyric acid, absorbs less than half as strongly, but the following member of the series, α-ketovaleric acid, absorbs more strongly than α-ketobutyric acid. There appears to be no progressive diminution of the intensity of absorption with increasing length of the carbon chain. It is interesting to note that the introduction of an additional —COOH group into α-ketobutyric acid, to give α-ketoglutaric acid, is accompanied by a diminution of the intensity of absorption. In order to produce the same absorption intensity as one molecular equivalent of pyruvic acid, about four equivalents of α-ketoglutaric acid are required. Lu [1939] reported a rather similar figure, 4·5. Table 3 shows the molecular quantities of the different α-keto-acids required to produce the same absorption intensity as 0·3 μmol. pyruvic acid.

Table 3. *The relative molecular concentrations of α-keto-acids required to produce a given absorption intensity*

	Drum reading	α-Keto-acid (μmol.)	Ratio		Drum reading	α-Keto-acid (μmol.)	Ratio
Pyruvic acid	0·360	0·300	1·0	α-Ketovaleric acid	0·360	0·615	2·1
α-Ketobutyric acid	0·360	0·781	2·6	α-Ketoglutaric acid	0·360	1·210	4·0

After having obtained the standard curves for the different α-keto-acids, it was then necessary to determine the recovery from deproteinized tissue extracts under the conditions to be observed experimentally.

A standard amount of the Na salt of the given α-keto-acid, contained in 1 ml. aqueous solution in a Dixon-Barcroft flask, and a water control were treated with 1 ml. 25 % trichloroacetic acid. 1 ml. of a brain suspension, equivalent to 300 mg. fresh brain, was then added, and after allowing 15 min. for complete precipitation of protein, the samples were filtered through 5·5 cm. filter papers (Whatman No. 42) into 10 ml. measuring cylinders. Each flask was rinsed with 2 ml. 5 % trichloroacetic acid three times, and the fluid used for washing the protein precipitate on the filter paper. After allowing to drain overnight, the volumes were made up to 10 ml. with distilled water. A 2 ml. aliquot was taken for estimating the amount of α-keto-acid by the method already described. At the same time, an equivalent amount of the Na salt of the α-keto-acid, not subjected to this extraction process, was estimated by the same method. A comparison of the absorption intensities of the two against the control gave a direct figure for the recovery of the α-keto-acid from the protein extract.

Table 4 shows three experiments, using pyruvic and α-ketobutyric acids, in which the recovery was determined. The average recovery is 97–99 %, a figure sufficiently near to the theoretical to make corrections unnecessary.

Table 4. *Recovery of pyruvic and α-ketobutyric acids from tissue extracts*

	Drum reading		
	Standard solution	Tissue extract	% recovery
Na pyruvate	0·572	0·555	97·0
Na α-ketobutyrate	0·386	0·375	97·2
	0·232	0·230	99·1

Returning to the curves in Fig. 2, it will be seen that those for pyruvic, α-ketobutyric, α-ketovaleric and α-ketoglutaric acids are of the same type. In all four cases the intensity of absorption is proportional to the concentration in very dilute solution; at higher concentrations, however, the absorption intensity increases less rapidly than would correspond with proportionality. The cause of this deviation from linearity is to be found in the fact that at higher concentrations the solution is deep red in colour, whereas at lower concentrations the tint is red-brown. It would therefore appear that on increasing the concentration, the maximum of the absorption band is moved towards the higher wave-lengths, so that the absorption intensity would no longer be expected to be directly proportional to the concentration. In order to show that this is probably the case, and that the cause is not associated with incomplete extraction of more concentrated solutions, the following experiment was designed. A solution of α-ketobutyric acid 2:4-dinitrophenylhydrazone in 10 % Na_2CO_3 was prepared as described. 3, 2 and 1 ml. of this extract were treated with 3 ml. 2*N* NaOH to develop the colour, and the volumes made up to 10 ml. The absorption intensities were compared against a control, and, as was to be expected, the relationship between concentration and absorption intensity was not linear. However, when the absorption intensities were converted into absolute amounts of α-ketobutyric acid, using the standard curve, they were seen to be almost in the exact ratio 3:2:1. The same was true using pyruvic acid 2:4-dinitrophenylhydrazone. The details of these experiments are given in Table 5.

Glyoxylic acid (curve *C*, Fig. 2) behaves differently from the other members of the α-keto-acid series, in that the relationship between concentration and absorption in-

Table 5. *The non-linear relation between concentration of α-keto-acid 2:4-dinitrophenylhydrazone and intensity of absorption*

	α-Keto-acid 2:4-dinitrophenylhydrazone (ml.)	Drum reading	Ratio	From standard curve. α-Keto-acid (μmol.)	Ratio
Na pyruvate	1	0·228	1·00	0·187	1·00
	2	0·442	1·93	0·383	2·05
	3	0·608	2·67	0·575	3·07
Na α-Ketobutyrate	1	0·108	1·00	0·170	1·00
	2	0·189	1·75	0·329	1·94
	3	0·268	2·48	0·519	3·05

tensity does not fall very far short of linearity, even at relatively high concentrations. This fact is also to be explained in terms of the wave-length of maximum absorption. Glyoxylic acid 2:4-dinitrophenylhydrazone gives with excess NaOH a bright orange-coloured solution, quite distinct from the red colours obtained with the other α-keto-acids. Moreover, the tint does not appear to vary appreciably with changes of concentration, so that an approximately linear relationship would be expected. It may be noted that glyoxylic acid differs in one further respect from its higher homologues, in that the colour complex of its 2:4-dinitrophenylhydrazone in excess alkali is rather unstable, the intensity of absorption falling by about 15% in an hour. This instability has been taken into account when plotting curve *C*, Fig. 2.

(5) *The utilization of pyruvic and α-ketobutyric acids by washed preparations of minced pigeon brain*

The primary object in attempting to develop a suitable method for the estimation of small amounts of α-keto-acids was to test directly the indications obtained by Long & Peters [1939] that pyruvic and α-ketobutyric acids were equally well utilized by a minced preparation of pigeon brain, a conclusion which had not been expected in view of previous work on α-keto-acid metabolism. The evidence put forward by Long & Peters was briefly as follows. The ultimate fate of pyruvic acid, when utilized by respiring minced pigeon brain, was known with some accuracy [Long, 1938]. A balance sheet accounting for 97% of the pyruvic acid disappearing showed that all previous observations could be explained on the assumption that three reactions were taking place:

(1) $CH_3CO.COOH + 2\frac{1}{2}O_2 \rightarrow 3CO_2 + 2H_2O$ ... ... 67·0%*

(2) $CH_3CO.COOH + \frac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$ ... ... 19·6%

(3) $CH_3CO.COOH + \frac{1}{2}H_2O \rightarrow \frac{1}{2}CH_3COOH + \frac{1}{2}CH_3CHOHCOOH + \frac{1}{2}CO_2$ 10·4%

Reaction (3) is an anaerobic process (dismutation), whereas (1) and (2) require the presence of oxygen. From the values quoted above, the calculated R.Q. was found to be in complete agreement with that determined experimentally by McGowan [1937]. Using the percentages given for the two aerobic reactions, the amount of pyruvic acid utilized by oxidative processes only was readily calculated from the observed net O_2 uptake.

The experimentally determined R.Q. for α-ketobutyric acid under the same conditions was found to be somewhat greater than 2·0, indicating that reaction (1) could not be taking part. In the same manner, the amount of α-ketobutyric acid utilized by oxidative decarboxylation only (reaction (2)) was calculated from the corresponding O_2 uptake. When identical samples of washed minced brain tissue were allowed to respire in pyruvate and α-ketobutyrate, side by side, and the separate net O_2 uptakes measured, it was calculated that the same amount of these two α-keto-acids was utilized by oxidative processes in a given period. Under anaerobic conditions it had been found that pyruvic and α-ketobutyric acids evolved the same volume of CO_2 in a given time, so that similarly

there was no doubt that they were also being utilized anaerobically at the same rate. This being so, it naturally followed that the total utilization of pyruvic and α-ketobutyric acids, whether due to aerobic or anaerobic processes, should also be identical. Since, however, a suitable method of estimating minute quantities of α-ketobutyric acid was not then available, it was not possible to test this point directly. Using the general method for α-keto-acid estimation, described above, this has now been done.

The experimental details for the preparation of the washed tissue mince and for the conduct of the respiration were exactly as described by Long & Peters [1939]. After mincing the cerebrum and optic lobes of three pigeons under ice-cold conditions, the brei was washed with ice-cold Ringer phosphate, *p*H 7·3, three times. Weighed portions were transferred to Dixon-Barcroft flasks containing *M*/50 pyruvate or α-ketobutyrate, and allowed to respire at 28 or 38°. The amount of each acid was determined in control flasks at the beginning of the respiration period, and also in the experimental flasks at the end of this period. The results of four experiments are shown in Table 6.

Table 6. *The utilization of pyruvic and α-ketobutyric acids by washed preparations of respiring minced pigeon brain*

Exp.	Temp.	Duration min.	Utilization μmol./g. tissue/hr. α-Ketobutyrate	Pyruvate
1	38	160	20·9	22·0
2	38	220	21·9	22·9
3	38	180	27·0	27·1
4	28	180	8·5	8·7

From Table 6 it is seen that within experimental error pyruvic and α-ketobutyric acids are equally rapidly utilized by washed preparations of minced pigeon brain. Thus the conclusions of Long & Peters [1939] are confirmed in detail.

Summary

1. Pyruvic, α-ketobutyric, α-ketovaleric, α-ketoglutaric and glyoxylic acids were found to give low values when estimated by the bisulphite-binding method, although acid $KMnO_4$ oxidation showed the samples to be quite pure.

2. A colorimetric method of estimating these α-keto-acids, based on the intensity of the red-brown colour obtained by the action of excess NaOH on the corresponding 2:4-dinitrophenylhydrazones, has been developed and found to be especially suitable for quantities of the order 0·1–1·0 μmol.

3. Using this method of estimation, it has been shown that pyruvic and α-ketobutyric acids are equally well utilized by a washed preparation of minced pigeon brain.

The author is greatly indebted to Prof. D. C. Harrison for much helpful criticism and advice.

REFERENCES

Bueding, E. & Wortis, H. [1940]. *J. biol. Chem.* **133**, 585.
Clift, F. P. & Cook, R. P. [1932]. *Biochem. J.* **26**, 1788.
Cohen, P. P. [1939]. *Biochem. J.* **33**, 1478.
Green, D. E., Needham, D. M. & Dewan, J. G. [1937]. *Biochem. J.* **31**, 2327.
Krebs, H. A. [1938]. *Biochem. J.* **32**, 108.
Lehnartz, E. [1928]. *Hoppe-Seyl. Z.* **179**, 1.
Long, C. [1938]. *Biochem. J.* **32**, 1711.
Long, C. & Peters, R. A. [1939]. *Biochem. J.* **33**, 759.
Lu, G. D. [1939]. *Biochem. J.* **33**, 249.
McGowan, G. K. [1937]. *Biochem. J.* **31**, 1627.
McGowan, G. K. & Peters, R. A. [1937]. *Biochem. J.* **31**, 1637.
Neuberg, C. & Ringer, M. [1915]. *Biochem. Z.* **71**, 226.
Peters, R. A. [1938]. *Biochem. J.* **32**, 2031.

95. The Component Fatty Acids of some Vegetable Seed Phosphatides

By **Thomas Percy Hilditch** and **Youssef Abdel Hamid Zaky**, *From the Department of Industrial Chemistry, University of Liverpool*

(*Received 24 August 1942*)

The component fatty acids of phosphatides present in soya beans and in rape seeds were determined [Hilditch & Pedelty, 1937] by the method of ester-fractionation. In both cases linoleic acid was found to be the predominating component, although all the acids present in the respective seed glycerides were also observed, to a greater or less extent, in the corresponding seed phosphatides; palmitic acid formed a greater proportion of the phosphatide than of the corresponding seed glyceride acids in both instances studied. A review of earlier observations on the composition of vegetable phosphatides, notably by Levene & Rolf [1925; 1926], by Suzuki & Nishimoto [1930] and Suzuki & Yokoyama [1931], was given in Hilditch & Pedelty's communication.

Specimens of phosphatide concentrates prepared from four other vegetable oils, groundnut, cottonseed, sunflower and linseed, have been supplied to us by Messrs J. Bibby and Sons, Ltd., of Liverpool, to whom we express our cordial thanks and appreciation. We have accordingly been able to obtain data concerning the component fatty acids present in these four seed phosphatides, of which a summary is here presented.

Experimental

Phosphatides from groundnuts

The phosphatide concentrate supplied was an emulsion consisting of approximately 50 % phosphatides, 25 % oil (glycerides) and 25 % water. The water and some mucilage was separated by extraction of the emulsion with 5 vol. of ether, and the dry mixture of phosphatides and glycerides was then boiled with five times its weight of acetone and the solution cooled at 0° overnight. This treatment was repeated several times until practically no material soluble in acetone at 0° remained and all the glycerides had been removed. After removing all traces of acetone from the final product in a vacuum at room temperature the groundnut phosphatides then formed a granular brownish coloured powder (sap. equiv. 288·23 i.v. (Wijs) 52·0). Although free from glycerides, the material still contained considerable proportions of non-fatty matter, and had P 2·7 %, N 1·2 % (molar ratio P/N 1·02 : 1).

The phosphatides (110 g.) were hydrolysed by refluxing with alcohol containing 0·5 % H_2SO_4 for 3 hr., after which sufficient KOH was added to neutralize the H_2SO_4 and give a 5 % solution of the alkali and the mixture was again refluxed for 3 hr. [cf. Hilditch & Pedelty, 1937]. On acidification of the recovered soaps, about 5 % of the product separated in the form of an amorphous non-fatty powder similar to that encountered in the case of soya bean phosphatides [Hilditch & Pedelty, 1937]; the liberated fatty acids were freed from residual traces of this substance by solution in light petroleum (B.P. 40–60°). The mixed fatty acids (59·7 g.) were separated by lead salt separation from alcohol into 'solid' (26·3 %) and 'liquid' (73·7 %) acids, each group being then converted into methyl esters which were fractionally distilled in a vacuum through an electrically heated and packed column with the results given in Table 1.

Table 1. *Methyl esters of acids from groundnut phosphatides*

From 'solid' acids				From 'liquid' acids			
No.	g.	Sap. equiv.	i.v.	No.	g.	Sap. equiv.	i.v.
S 1	3·03	273·8	1·7	L 1	2·31	281·3	46·4
S 2	2·75	275·0	2·7	L 2	3·46	294·6	101·4
S 3	3·03	285·5	32·6	L 3	4·15	295·0	115·6
S 4	3·50	330·0	27·5	L 4	4·41	295·6	116·4
S 5	1·80	445·0*	18·0	L 5	4·02	297·0	112·6
				L 6	5·16	298·4	112·6
				L 7	2·95	298·7	108·0
				L 8	3·80	408·0*	82·9
	14·11				30·26		

* Sap. equiv. of esters, freed from unsaponifiable matter, S 5, 414·2; L 8, 302·0.

The acids from fraction S 4 yielded on crystallization from ethyl acetate crystals which melted at 80° (mixed with behenic acid, M.P. 81°); the residual unsaturated acids recovered from the mother liquors had sap. equiv. 285·0 and therefore consisted substantially of oleic acid (282).

The acids from fractions L 4 and L 6 were brominated in anhydrous ether at 60°. Those from L 4 gave no ether-insoluble bromo-adducts, but deposited a crystalline acid from light petroleum M.P. 114° (unchanged when mixed with tetrabromostearic acid M.P. 114°, from seed fat linoleic acid). Those from L 6 gave small amounts of solid ether-insoluble bromo-derivatives which melted indefinitely with decomposition somewhat above 200°, thus indicating the presence of polyethenoid acids of the C_{20} and C_{22} series. Thiocyanogen values of the ester-fractions of the 'liquid' group confirmed that linoleic acid was not present in the groundnut phosphatide acids.

The final composition of the groundnut phosphatide acids, deduced from the above data, is given in Table 2.

Table 2. *Component acids of groundnut phosphatides*

	'Solid' acids S (26·3 %)	'Liquid' acids L (73·7 %)	Total	Component acids % (wt.)	Component acids % (mol.)
Palmitic	11·20	4·49	15·69	16·2	17·8
Stearic	2·73	—	2·73	2·8	2·8
C_{20}, C_{22}, C_{24} As C_{26}	4·47	—	4·47	4·6	3·9
	2·45	—	2·45	2·5	1·8
Oleic	5·21	40·61	45·82	47·1	47·2
Linoleic	—	22·04	22·04	22·7	22·8
Unsaturated C_{20-22}	—	4·05	4·05	4·1	3·7
Unsaponifiable	0·24	2·51	2·75	—	—

Phosphatides from cottonseed

The cottonseed phosphatide concentrate, supplied in the form of an emulsion with glycerides and water, was treated in the same way as the groundnut phosphatide concentrate described above, and a granular purified phosphatide (still containing some non-fatty matter) was finally obtained (P 2·8 %, N 1·6 %; molar ratio P/N 0·8 : 1). The cottonseed phosphatides (180 g.) yielded on hydrolysis 85·8 g. (48 %) of mixed fatty acids, which were resolved by lead salt separation from alcohol into 24·5 % 'solid' and 75·5 % 'liquid' acids. Fractionation of the corresponding methyl esters led to the results given in Table 3.

The final composition deduced for the fatty acids in the cottonseed phosphatides is given in Table 4.

Table 3. *Methyl esters of acids from cottonseed phosphatides*

From 'solid' acids				From 'liquid' acids			
No.	g.	Sap. equiv.	i.v.	No.	g.	Sap. equiv.	i.v.
S 1	3·29	277·3	0·6	L 1	3·48	272·8	52·4
S 2	3·43	280·2	0·6	L 2	3·83	289·4	120·4
S 3	4·73	280·2	7·0	L 3	3·94	291·5	141·0
S 4	1·85	338·2	8·8	L 4	5·18	294·1	146·6
				L 5	6·06	294·0	145·0
				L 6	6·22	294·8	145·0
				L 7	5·27	295·9	145·3
				L 8	6·04	296·1	143·5
				L 9	5·45	297·0	143·0
				L 10	7·30	297·1	140·0
				L 11	6·94	(462·5)*	(53·0)*
	13·30				59·71		

* Sap. equiv. of esters, freed from unsaponifiable matter, S 4, 322·0; L 11, 318·7. The residue of 11 was lost owing to enemy action; the sap. equiv. and i.v. used were obtained in a separate distillation of a small portion of the methyl esters from the total phosphatide acids. These values may therefore be not wholly accurate, and the actual proportion of unsaturated C_{20-22} acids present may be somewhat different from that shown in Table 4.

Table 4. *Component acids of cottonseed phosphatides*

	'Solid' acids S (24·5%)	'Liquid' acids L (75·5%)	Total	Component acids % (wt.)	Component acids % (mol.)
Palmitic	13·48	3·34	16·82	17·3	18·9
Stearic	7·03	—	7·03	7·3	7·1
Arachidic	2·73	—	2·73	2·8	2·5
Hexadecenoic	—	1·46	1·46	1·5	1·7
Oleic	1·09	18·56	19·65	20·3	20·0
Linoleic	—	43·07	43·07	44·4	44·2
Unsaturated C_{20-22}	—	6·21	6·21	6·4	5·6
Unsaponifiable	0·17	2·86	3·03	—	—

Phosphatides from sunflower seed

The quantity of sunflower seed phosphatides available was small, and only 40 g. of purified material were obtained for component acid analysis. The fatty acids obtained by hydrolysis of the phosphatides gave, on lead salt separation from alcohol, 32·9% 'solid' and 67·1% 'liquid' acids. Fractionation of the corresponding methyl esters gave the data summarized in Table 5.

Table 5. *Methyl esters of acids from sunflower seed phosphatides*

From 'solid' acids				From 'liquid' acids				
No.	g.	Sap. equiv	i.v.	No.	g.	Sap. equiv.	i.v.	CNS. V
S 1	2·74	278·2	15·6	L 1	3·61	290·4	129·6	73·4
S 2	2·66	353·0*	18·6	L 2	3·66	294·0	153·3	88·4
				L 3	3·50	297·7	155·8	90·5
				L 4	3·00	364·1*	130·0	—
	5·40				13·77			

* Sap. equiv. of esters, freed from unsaponifiable matter, S 2, 314·2; L 4, 303·8.

The thiocyanogen values of the 'liquid' esters did not indicate the presence of linolenic acid, and the data calculated for the component acids are therefore as shown in Table 6.

The component acids of the sunflower seed oil (glycerides) corresponding with the phosphatides examined were also determined, with the final results given in Table 7.

The oil had sap. equiv. 295·0, i.v. (Wijs) 138·2 and contained 1·0 % of free fatty acids (as oleic).

Table 6. *Component acids of sunflower seed phosphatides*

	'Solid' acids S (32·9 %)	'Liquid' acids L (67·1 %)	Total	Component acids % (wt.)	Component acids % (mol.)
Palmitic	11·37	2·71	14·08	14·7	16·1
Stearic	4·87	—	4·87	5·1	5·0
Arachidic	9·10	—	9·10	9·5	8·6
Oleic	5·71	12·69	18·40	19·3	19·2
Linoleic	—	43·87	43·87	45·9	46·3
Unsaturated C_{20-22}	—	5·28	5·28	5·5	4·8
Unsaponifiable	1·85	2·55	4·40	—	—

Table 7. *Component acids of sunflower seed oil*

	'Solid' acids S (6·8 %)	'Liquid' acids L (93·2 %)	Total	Component acids % (wt.)	Component acids % (mol.)
Palmitic	2·79	2·79	5·58	5·6	6·2
Stearic	2·13	—	2·13	2·2	2·1
Arachidic	0·92	—	0·92	0·9	0·8
Oleic	0·90	23·95	24·85	25·1	24·8
Linoleic	—	65·68	65·68	66·2	66·1
Unsaponifiable	0·06	0·78	0·84	—	—

Other workers [Baughman & Jamieson, 1922; Branke & Gutt, 1935] have given the component acids (% wt.) of sunflower seed oil as follows:

	Baughman & Jamieson	Branke & Gutt
Palmitic	3·9	1·6
Stearic	2·9	6·9
Arachidic	—	—
Oleic	34·2	26·6
Linoleic	59·0	64·9

Phosphatides from linseed

Unfortunately the amount of phosphatides from this interesting source was extremely small (20 g.). The acids obtained after hydrolysis of the phosphatides furnished, after lead salt separation from alcohol, 2·6 g. (20·4 %) 'solid' and 10·3 g. (79·6 %) 'liquid' acids. The 'solid' acids were converted into methyl esters, which had sap. equiv. 286·1 (freed from unsaponifiable, 285·4), i.v. 6·6. The methyl esters of the 'liquid' acids were fractionally distilled in the usual way with the following results:

No.	g.	Sap. equiv.	i.v.	CNS. V.
L 1	2·30	286·2	126·4	86·7
L 2	3·38	293·0	154·2	104·8
L 3	3·12	295·7	156·8	106·7
L 4	1·41	480·0*	131·9	71·9
	10·21			

* Esters in L 4, freed from unsaponifiable matter, sap. equiv. 304·0.

The esters of unsaturated C_{18} acids present in these fractions were calculated, from the iodine and thiocyanogen values, as mixtures of methyl oleate, linoleate, and linolenate; the thiocyanogen values for pure methyl linoleate and linolenate were taken respectively as 91·8 and 154·7 [Hilditch & Murti, 1940]. The final results for the linseed phosphatide component acids are given in Table 8.

Table 8. *Component acids of linseed phosphatides*

	'Solid' acids S (20·4%)	'Liquid' acids L (79·6%)	Total	Component acids % (wt.)	Component acids % (mol.)
Palmitic	8·68	2·17	10·85	11·3	12·3
Stearic	10·19	—	10·19	10·6	10·4
Hexadecenoic	—	3·33	3·33	3·5	3·8
Oleic	1·47	30·64	32·11	33·6	33·1
Linoleic	—	19·53	19·53	20·4	20·4
Linolenic	—	16·64	16·64	17·4	17·4
Unsaturated C_{20-22}	—	3·05	3·05	3·2	2·8
Unsaponifiable	0·06	4·24	4·30	—	—

Discussion and summary

Including the soya bean and rape seed phosphatides previously studied, the component fatty acids of the phosphatides from six different species of seeds have now been determined. The proportions of the various acids present, in the phosphatides and also in the corresponding seed glycerides (for which typical recorded analyses are included), are shown in Table 9.

Table 9. *Component acids of seed phosphatides and glycerides* (*wt.* %)

	Cotton-seed	Sunflower seed	Rape seed	Soya-bean	Ground-nut	Linseed
			(i) Phosphatides			
Myristic	—	—	0·8	—	—	—
Palmitic	17·3	14·7	8·3	11·7	16·2	11·3
Stearic	7·3	5·1	—	4·0	2·8	10·6
Arachidic	2·8	9·5	—	1·4	—	—
C_{20}, C_{22}, C_{24}	—	—	1·5	—	4·6	—
As C_{26}	—	—	—	—	2·5	—
Hexadecenoic	1·5	—	2·1	8·6	—	3·5
Oleic	20·3	19·3	22·4	9·8	47·1	33·6
Linoleic	44·4	45·9	42·2	55·0	22·7	20·4
Linolenic	—	—	Trace	4·0	—	17·4
Unsaturated C_{20-22}	6·4	5·5	—	5·5	4·1	3·2
Erucic	—	—	22·7	—	—	—
			(ii) Glycerides			
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
Myristic	1·4	—	—	0·3	—	0·2
Palmitic	23·4	5·6	2	9·8	8·3	5·4
Stearic	1·1	2·2	—	2·4	3·1	3·5
Arachidic	1·3	0·9	—	0·9	—	0·6
C_{20}, C_{22}, C_{24}	—	—	1	—	6·6	—
Hexadecenoic	2·1	—	—	0·5	—	—
Oleic	22·9	25·1	17	28·9	56·0	18·8
Linoleic	47·8	66·2	29	50·7	26·0	24·2
Linolenic	—	—	—	6·5	—	47·3
Erucic	—	—	51	—	—	—

(*a*) Hilditch & Maddison [1940]; (*b*) present work; (*c*) Hilditch & Paul [1935]; (*d*) Hilditch & Jasperson [1939]; (*e*) Hilditch & Jasperson [1938]; (*f*) Griffiths & Hilditch [1934]; Hilditch & Murti [1940].

The data presented in this paper largely confirm but also extend the conclusions suggested by Hilditch & Pedelty [1937] from the two seed phosphatides then studied. In all six instances the range of fatty acids in the phosphatides is qualitatively the same as that in the corresponding glycerides, but the proportions of the component acids vary widely as between the two types of seed lipids. The features most general throughout the six phosphatides appear to be the consistent occurrence of a minor proportion (3–6 %) of highly unsaturated acids of the C_{20} and C_{22} series (which are not present in seed

glycerides), and the presence of more saturated acids in the phosphatides than in the glycerides.

The presence of appreciable, though small, amounts of the highly unsaturated acids of higher molecular weight than C_{18} (possibly arachidonic, possibly mixtures of polyethenoid C_{20} and C_{22} acids) seems to be a characteristic of all natural phosphatides from vegetable or animal sources.

The increased proportions of saturated acids in phosphatides as compared with glycerides from the same site is also common to both vegetable and animal lipids. In the six seed phosphatides the increase is chiefly in the proportion of palmitic acid (the characteristic saturated acid of all vegetable lipids), although stearic acid also occurs to a larger degree in some of the phosphatides as compared with the corresponding glycerides. Again it may be noticed that in cottonseed, the glycerides of which contain a comparatively high proportion of saturated acids, the total amount of the latter in the phosphatide acids is not materially increased whilst the proportion of palmitic acid has decreased (from 23 to 17 %).

Hexadecenoic acid, which is found as a minor component in all animal phosphatides, was observed to be similarly present in rape seed phosphatides and to a somewhat larger extent in soya bean phosphatides; but the extended study suggests that on the whole it is a definitely minor component of seed phosphatide acids. Indeed, no evidence of its presence was observed in the phosphatides from groundnuts and sunflower seed.

The most abundant components of the seed phosphatides are linoleic and oleic acids. In our earlier communication it was suggested that linoleic acid might be the most important component of plant phosphatide acids, exceeding in its proportions those of oleic or other acids, with the ratio of linoleic to oleic acids higher in the phosphatides than in the corresponding glycerides. In four of the six instances now available, linoleic is the most abundant acid and forms 42–55 % of the total fatty acids. In these cases it also occurs in considerably higher proportions than oleic acid; but only in rape seed is the amount of linoleic acid notably higher in the phosphatides than in the glycerides. In the soya bean it is somewhat higher, in cottonseed, linseed and groundnuts somewhat lower, and in sunflower seed it is much lower in the phosphatides than in the glycerides.

In cottonseed and groundnuts the relative proportions of linoleic and oleic acids are much the same in both glycerides and phosphatides. In sunflower seed and in linseed the ratio of linoleic acid to oleic acid in the phosphatides is definitely lower than in the glycerides. Linseed stands somewhat apart from the rest in that the relative proportions of linolenic, linoleic and oleic acids in the glycerides are reversed in the phosphatides; in the latter oleic acid is the most, and linolenic the least, abundant of the three acids. In the light of the further instances now investigated, the earlier suggestion that linoleic is the most important component of seed phosphatides must therefore be replaced by the statement that linoleic acid (identical with seed glyceride linoleic acid) has been found to be an invariable major component of seed phosphatides, and that it frequently, but not always, forms 45 % or more of the total fatty acids.

From the six instances now studied (which cover a fairly wide range of botanical families) the following generalizations may be tentatively suggested: (i) seed phosphatides contain characteristic, although minor proportions of highly saturated C_{20} and C_{22} acids which are not present in the corresponding glycerides; (ii) acids (notably palmitic) of the saturated series form a greater proportion of seed phosphatide than of seed glyceride fatty acids; (iii) all the acids present in any seed glyceride are also found in the corresponding seed phosphatide; (iv) linoleic acid is on the whole the most characteristic acid of seed phosphatides and, although in two instances out of the six investigated it amounts to only 20–25 % of the total fatty acids, in the majority it forms 45–55 % of the total phosphatide fatty acids.

REFERENCES

Baughman, W. F. & Jamieson, G. S. [1922]. *J. Amer. chem. Soc.* **44**, 2952.
Branke Y. V. & Gutt, E. F. [1935]. *Bull. Far East Branch Acad. Sci. U.S.S.R.* **13**, 17.
Griffiths, H. N. & Hilditch, T. P. [1934]. *J. Soc. chem. Ind., Lond.*, **53**, 13T.
Hilditch, T. P. & Jasperson, H. [1938]. *J. Soc. chem. Ind., Lond.*, **57**, 363.
—— —— [1939]. *J. Soc. chem. Ind., Lond.*, **58**, 187.
Hilditch, T. P. & Maddison, L. [1940]. *J. Soc. chem. Ind., Lond.*, **59**, 162.
Hilditch, T. P. & Murti, K. S. [1940]. *Analyst*, **65**, 437.
Hilditch, T. P. & Paul, H. [1935]. *J. Soc. chem. Ind., Lond.*, **54**, 331T.
Hilditch, T. P. & Pedelty, W. H. [1937]. *Biochem. J.* **31**, 1964.
Levene, P. A. & Rolf, I. P. [1925]. *J. biol. Chem.* **62**, 759; **65**, 545.
—— —— [1926]. *J. biol. Chem.* **68**, 285.
Suzuki, B. & Nishimoto, U. [1930]. *Proc. imp. Acad. Japan*, **6**, 262.
Suzuki, B. & Yokoyama, Y. [1931]. *Proc. imp. Acad. Japan*, **7**, 12, 226.

96. Phosphatides from Oil Seeds

By **Bruno Rewald**, *Brenchley, Penn Road, Beaconsfield*

(*Received 24 August 1942*)

All oil-producing seeds contain phosphatides, but so far only a few of these seeds have been examined carefully with regard to their phosphorus-containing glycerides. There are many publications about the phosphatides of soya beans, and a few other phosphatides have been also examined (e.g. rape seed), but there seems to be very little known about the phosphatides of other important oil seeds, especially those of groundnuts, cottonseed, linseed or sunflower seed. For this reason, experiments have been carried out to extract these phosphatides from the raw materials, or to separate them from the sludge of commercially manufactured oils. As it is well known that the phosphatides of the vegetable kingdom are always mixtures of choline and aminoethanol phosphatides, a separation of these two groups was carried out, and the percentage ratio determined.

Oil seeds are usually crushed in expeller presses or similar machines, and the crude oil thus obtained contains a certain amount of phosphatides which separate out, with other impurities, after the oil has been kept in storage for some time. These sludges have been one source of raw material for the extraction and separation of the phosphatides, although only a certain percentage, usually no more than half of the phosphatides present in the seeds, is found in the oil; another, and sometimes a very large amount, of these phosphatides remains in the press cake. It seems that the phosphatides are distributed in the oil-containing seed in two different forms: in one form they can be regarded as 'free' phosphatides, and here they are dissolved in the oil and therefore are pressed out together with the oil and later settle out in the sludge; in the other case they seem to be 'physically' linked, usually to the proteins and sugars. It is very well known that egg yolk for instance contains most of its lecithin in a combined form which cannot be extracted with the ordinary solvents, light petroleum, benzene, trichlorethylene etc.; only by the use of alcohol can the lecithin be freed from the proteins and later be extracted without any difficulty. The same phenomena occur in seeds. The phosphatides remaining in the press cakes cannot be extracted with any of the ordinary solvents in absence of alcohol. For this reason the cakes were exhaustively extracted with a mixture of alcohol and benzene (20 : 80). Such a mixture is necessary because of the presence of large quantities of phosphatides of the kephalin type, which are not soluble even in boiling alcohol, and require a solvent in which the phosphatides can be dissolved later. In previous cases [Rewald, 1936; 1937 *a*, *b*] this particular mixture gave very satisfactory results.

The extraction of the phosphatides was always carried out in the same way. The sludge usually contains a high amount of moisture, besides large quantities of oil and impurities (husks, etc.). This sludge is treated with a large quantity of acetone, which removes not only the water but also the oil, sterols, pigments, etc. The remaining paste is dried and dissolved in light petroleum. It always takes some time before the very fine powder has settled out and the clear solution can be filtered. This solution contains the phosphatides and a certain amount of oil. It is treated with an excess of acetone, and the flocculated phosphatides are separated and dried in a vacuum. This procedure has to be repeated once or twice. The phosphatides must be stored in a vacuum desiccator and the adherent solvent evaporated very carefully. Phosphatides in a pure state absorb moisture very quickly and decompose in a short time if exposed to the open air.

These phosphatides are not absolutely pure. They still contain a certain amount of carbohydrate, mostly disaccharides, which are very difficult to remove. It is interesting that these mixtures of phosphatides and sugars are easily soluble in nearly all of the fat solvents in which sugars are otherwise absolutely insoluble. This sugary matter can be mostly removed by prolonged shaking of the solution in light petroleum with water, but the emulsions formed and the losses are so great, that the preparation of sugar-free phosphatides not only takes a very long time, but also needs rather large quantities of material. The final purification was not carried out in the following cases, which probably accounts for the somewhat low phosphorus figures observed.

The extraction of the phosphatides from the finely ground oil cakes was carried out as follows. The cake was first extracted with light petroleum, to remove any oil still present. This oil, as expected, contained only traces of phosphatides. The oil-free powder was then repeatedly boiled, under reflux, with a mixture of alcohol and benzene. The solvent was evaporated, and the dry residue extracted with light petroleum. As is always the case when alcohol is used, this residue contained large quantities of sugary matter. The clear solution was then treated with acetone, which, as in the previous case, flocculated the phosphatides. The purification by redissolving in light petroleum, etc. was carried out twice.

The phosphatides, extracted from the sludge or the press cake, were then separated into the two main fractions, lecithin and kephalin, by using boiling alcohol, which dissolves the lecithin but not the kephalin. Occasionally a third fraction could be separated, soluble in hot alcohol but insoluble in cold; it could not be determined whether this fraction is a pure compound or a mixture, but it was found that it could not be resolved further by treating it many times with boiling alcohol.

The appearance of the phosphatides proves that they must contain a large amount of kephalin in addition to lecithin, since they are mostly somewhat hard solids, whilst lecithins are soft and pasty. This is confirmed by analysis, which in most cases indicate that only one-third of the total phosphatides belong to the lecithin type.

The results of the experiments can be seen from the tables.

Table 1 gives the percentages of phosphorus found in the lecithin and kephalin separated from the sludges of the different oils pressed from the seeds. It will be seen that the lecithin figures are on the low side, which may be due to the presence of carbohydrates; the kephalin figures are in most cases more normal. Only the linseed and the sunflower seed products are out of range, but linseed is a drying oil and it must be borne in mind that decomposition may have occurred, which might well influence the solubility.

Table 1. *Phosphatides in seed oil sludges*

Oil	Lecithin (alcohol-soluble) % P	Kephalin (alcohol-insoluble) % P	Soluble in hot, insoluble on cold, alcohol % P
Groundnut	3·64	4·26	3·52
Sesame	3·29	4·34	3·90
Cottonseed	3·52	4·00	—
Linseed	3·35	3·00 (?)	—
Sunflower seed	3·08	2·94 (?)	—

Table 2 shows the data for the phosphatides extracted from the cakes. It is remarkable that nearly always the same amount (about 0·5 %) of phosphatides remains in the cake. The quantities of oil which remain in the cake are 6–8 %, and the phosphatides are only present in small amounts in these oils. For comparison linseed itself was also treated in the same way; here the quantity of oil is naturally high, but the alcohol-benzene extract is nearly the same, and the phosphatide isolated also has the same composition (3·71 and 3·93 % P).

Table 2. *Phosphatides in oil press-cakes*

Seed-cake	Light petroleum extract: Extract %	Light petroleum extract: P content %	Alcohol-benzene extract %	Acetone-insoluble: P %	Acetone-insoluble: Lecithin equivalent in cake %
Groundnut	8·43	Traces	1·34	3·77	0·57
Cottonseed	7·19	0·20	1·31	3·59	0·58
Linseed	6·24	0·41	3·82	3·71	0·41
Linseed meal (not pressed)	38·00	0·02	2·81	3·93	—

Table 3 gives the distribution of lecithin and kephalin in the phosphatides.

Table 3. *Ratio of lecithin and kephalin in seed phosphatides*

	Lecithin %	Kephalin %
Groundnut	35·7	64·3
Sesame	52·2	40·6*
Cottonseed	28·8	71·2
Linseed	36·2	63·8
Sunflower	38·5	61·5

* 7·2% soluble in hot, insoluble in cold, alcohol.

I am indebted to Messrs J. Bibby and Sons, Ltd., of Liverpool for the different raw materials used in these investigations.

REFERENCES

Rewald, B. [1936]. *Food*, **6**, 7.
—— [1937*a*]. *J. Soc. Chem. Ind., Lond.*, **56**, 77T.
—— [1937*b*]. *J. Soc. Chem. Ind., Lond.*, **56**, 403T.

97. X-Ray Diffraction Studies in Iodinated Amino-acids and Proteins

By **M. Spiegel-Adolf, R. H. Hamilton, Jr.** and **G. C. Henny**, *From the Department of Colloid Chemistry, D. J. McCarthy Foundation, the Departments of Biochemistry and Physics, Temple University School of Medicine, Philadelphia, Penna.*

(*Received 8 August 1942*)

The biological activity of some of the iodinated amino-acids and proteins gives rise among others to the additional problem 'does the process of iodinating the protein also change molecular configuration so as to produce physiological activity?' [Salter, 1940]. X-ray diffraction studies of the various stages of iodination in aromatic amino-acids and in proteins seemed therefore promising.

Tyrosine and thyroxine and some of the intermediate products were investigated. Natural iodinated globulins, thyroglobulins, were made available to us through the kindness of Dr J. F. McClendon. Besides, we were able to study an artificially iodinated protein, iodocasein, which according to findings of Ludwig & Mützenbecher [1939], confirmed by Harington & Pitt Rivers [1939], is physiologically active and contains thyroxine. Finally, a plastein derived from the above iodocasein has been prepared by one of us (R. H. H.).

Technique and material

The X-ray diffraction apparatus used in our measurements has been described before [Spiegel-Adolf & Henny, 1939]. The only modification of the method was made necessary by the varying content of iodine with its high opacity to X-rays. Although the iodine content practically did not interfere with the X-ray diffraction of natural and artificial iodoproteins, it made analogous studies impossible in iodinated aromatic amino-acids. Therefore, following Clark [1940], admixtures of gums (tragacanth and acacia) were used, corresponding to a ratio of 8 : 1 in terms of iodine. The X-ray diffraction patterns of tragacanth and acacia are different, so that by using either one in different exposures, besides taking control diffraction patterns with pure gums, it is easy to eliminate diffraction lines pertaining to the gums. A uniform exposure time of 2 hr. was used.

No comparable data seem to be available for our iodinated material. Therefore KI, the powder data of which have been reported by Hanawalt, Rinn & Frevel [1938], was studied in an appropriate mixture with charcoal. In Table 1 our results are presented in comparable form with those of Hanawalt *et al.* They seem to agree sufficiently.

Table 1. *KI 1 part, charcoal 6 parts. Comparison of molecular spacings*

Line, in order of intensity	Our value Å.	Published value Å.	Line, in order of intensity	Our value Å.	Published value Å.
1	3·525	3·53	4	2·147	2·13
2	2·520	2·50	5	2·045	2·03
3	4·09	4·08			

3:5-Diiodotyrosine was prepared by direct iodination of *l*-tyrosine (Eastman) in strong ammonia solution at 0° with the theoretical amount of iodine in KI solution; the material was recrystallized from 33 % acetic acid and finally from water. The iodine content is 58·7 %.

3:5-Diiodothyronine was prepared by the method of Harington & Barger [1927] as modified by Harington & McCartney [1927]. After recrystallization from alcohol the m.p. was 241°, and the iodine content 48·3 %.

Two different commercial brands of thyroxine were used, a preparation of natural thyroxine (Squibb) and a synthetic preparation from Roche. Three samples of human thyroglobulins were available which had been prepared from the original glands by Cavett, Rice & McClendon [1935] by the method of Cavett & Seljeskog [1933]. The origins of our thyroglobulin preparations, their iodine, thyroxine and phosphorus content as originally evaluated are summarized in Table 2.

Table 2. *Data on origin and chemical analyses of thyroglobulins [Cavett, Rice & McClendon]*

Thyroglobulin from	Colloid goitre	Exophthalmic goitre	Normal gland
Thyroxine %	0·0046	0·153	0·382
Total iodine %	0·031	0·40	0·57
$\frac{\text{Thyroxine iodine}}{\text{Total iodine}}$	0·11	0·25	0·45
Phosphorus %	0·032	0·035	0·025

These preparations still contained amounts of ammonium sulphate which interfered with the X-ray diffraction patterns. Part of the preparations were freed from the salt contaminations as described by Cavett & Seljeskog [1933] using heat precipitation, and subsequent washing of the precipitates. To avoid denaturation the remaining preparations were dissolved in water, dialysed against flowing distilled water and finally electrodialysed until free from electrolytes. The preparations were then dried in a desiccator over P_2O_5 and ground in an agate mortar. Iodinated casein was prepared by direct iodination of casein [Pfanstiehl, Casein Pure, lot 552). The casein was dissolved in strong ammonia (sp. gr. about 0·93). The solution was cooled to 0° and with good stirring the amount of strong aqueous I_2-KI was added that was calculated to be required for complete iodination of all tyrosine groups. The ammonia was evaporated off in a vacuum, and the iodinated product was reprecipitated several times at its isoelectric point. (Found: I, 5·2; N, 12·78 %; I/N ratio 0·407.) Assuming N 15·7 % and tyrosine 4·5 % for casein, the theoretical I/N ratio is 0·401. Plastein from iodinated casein was prepared in a manner similar to that of Wasteneys & Borsook [1924–5]. Iodinated casein was dissolved in dilute HCl (*p*H 1·96) and digested with pepsin. The acidity was adjusted to *p*H 4·0, and insoluble material filtered off. The solution was concentrated at low temperature, and a large amount of pepsin in concentrated solution was added. The precipitate that developed during 2 hr. at 60° was washed three times in the centrifuge with water and then dried. (Found: I, 6·74; N, 11·81 %; I/N ratio = 0·571.)

Results

The findings of X-ray diffraction studies in iodinated aromatic amino-acids and derivatives are summarized in Table 3.

These results show that iodination of tyrosine and diiodothyronine notably alters the X-ray diffraction patterns of these compounds. Two diffraction rings (corresponding to spacings of 4·35 and 5·03 Å. respectively) are strong in the tyrosine and weak in the diiodotyrosine diffraction patterns. Conversely at least two new diffraction rings (corresponding to spacings of 3·92 and 13 Å.) become manifest in diiodotyrosine and persist in diiodothyronine and thyroxine. One of these rings (corresponding to a spacing of 3·92 Å.) may have direct relation to iodine since it not only exists in the diffraction patterns of the above mentioned organic compounds but also in mixtures of tragacanth and KI, but not in mixtures of KI and charcoal. The diffraction pattern of diiodothyronine contains many more rings than the one of thyroxine. A moderately strong ring corresponding to a spacing of 5·57 Å., and two faint rings corresponding to spacings 2·13 and 2·36 Å., which occur in the former compound are missing from the thyroxine

Table 3. *X-ray diffraction patterns of iodinated aromatic amino-acids and controls*

(Interplanar spacings in Ångström units, lines in order of intensity.)

	Tyrosine*	Diiodo-tyrosine	Diiodo-thyronine	Thyroxine		Tragacanth	KI
1	4·35	3·92	5·57	3·97	1	9·78	3·53
2	5·03	12·97	13·72	12·28	2	5·22	2·50
3	9·78	3·03	3·86	3·57	3	4·22	3·92
4	5·69	5·03	4·22	3·30	4	—	2·15
5	2·13	4·35	4·75	4·36	5	—	2·05
6	2·28	7·86	9·78	5·02	6	—	2·75
7	3·12	2·78	3·06	4·66			
8	2·67	3·41	2·63	2·67			
9	3·33	2·55	3·62	—			
10	—	—	3·33	—			
11	—	—	2·36	—			
12	—	—	2·13	—			

* Tyrosine contains the same amount of tragacanth as diiodotyrosine.

diffraction pattern. Conversely the latter pattern only shows a strong ring corresponding to a spacing of 3·57 Å. These findings seem to indicate that iodination of aromatic amino-acids is probably accompanied by some kind of molecular rearrangement.

Neither the naturally iodinated thyroglobulins nor the artificially iodinated casein show any differences in the X-ray diffraction patterns which would distinguish them from the corresponding iodine-free proteins. In both instances the two diffuse rings correspond to spacings of 4·5 and 10·19 Å. Thyroglobulin was especially compared with pseudoglobulin which is precipitated by similar ammonium sulphate concentrations. On account of its water-solubility Heidelberger & Pedersen [1935] call thyroglobulin a pseudoglobulin. The undenatured thyroglobulins show diffraction patterns free from any back-bone sharpening. (According to Spiegel-Adolf & Henny [1941; 1942] these findings do not imply that the thyroglobulin is necessarily undenatured; they only suggest that a denaturation is possibly different from changes brought about by heating.) The well-known heat-precipitability of thyroglobulin is accompanied as in other proteins by a sharpening of the back-bone reflexion. This seems interesting in view of the large molecular weight of thyroglobulin [Heidelberger & Pedersen, 1935], which apparently does not interfere with the process of heat-denaturation.

Neither of the two groups of iodinated proteins shows any diffraction rings found in the X-ray diffraction patterns of diiodothyronine or thyroxine. It seems probable that the small amount of thyroxine present in thyroglobulins (even those richest in thyroxine do not contain over 0·38 %) may account for these negative findings. In order to test this assumption X-ray diffraction pictures were taken of artificial mixtures of thyroglobulins and thyroxine containing 4, 2 and 1 % of the latter. While samples containing 4 % thyroxine showed clearly some of the thyroxine diffraction lines, especially the ones corresponding to spacings of 3·57 and 3·97 Å., only traces of these lines could be recognized in the 2 % sample. The 1 % sample did not show any indications of thyroxine admixtures. We do not suggest that our mixtures are identical with physiological compounds of higher thyroxine content. Nevertheless it seems probable that the X-ray diffraction method as handled by us is not sensitive enough to trace minute amounts of thyroxine present in normal and pathological thyroglobulins.

The negative findings in the case of iodocasein were perhaps even more surprising in view of the high iodine content of our preparation. Assuming all the iodine to be in the form of diiodotyrosine, an iodine content of 5·2 % corresponds to a diiodotyrosine content of 8·87 %. Artificial mixtures of this amount of diiodotyrosine and casein show very clearly the characteristic diffraction pattern of the former substance superimposed on the protein diffraction pattern. It seems therefore that the molecular arrangement of the iodocasein is such as to prevent the spacings of the free diiodotyrosine from becoming

manifest. A similar changed state or relationship of the atoms may well bear on the question raised by Harington & Pitt Rivers as to how it is possible for thyroxine to be produced by iodination of a protein and subsequent hydrolysis. Such a synthesis is impossible in vitro by action of iodine upon thyronine.

Our method was more successful in tracing differences existing between plastein derived from iodocasein and its mother-substance. According to our results plastein shows that the diameter of the inner diffraction ring, corresponding to the side-chain spacing, is 2 mm. smaller than the inner ring of the iodocasein diffraction pattern. Besides, the outer diffraction ring, corresponding to the back-bone spacing, is sharp in plastein and diffuse in iodocasein. In order to get an objective evaluation of these differences, relative light transmission graphs and exact centre determinations of these films were made with the help of our photoelectric microdensitometer [Spiegel-Adolf & Peckham, 1940]. The differences observed in the X-ray diffraction patterns of plastein can be probably explained by differences in hydration [Katz, 1934]. Our plastein shows appreciable physiological activity and chemically a much higher thyroxine content than iodocasein. Salter [1935] who studied a plastein resynthesized from thyroid peptones could at least trace differences of physicochemical nature between the artificial and the original products. Our findings seem to add additional material to the arguments of authors, who like Flosdorf [1941], deny the identity of plasteins with their mother substance.

SUMMARY

1. X-ray diffraction studies were made of tyrosine, 3:5-diiodotyrosine, 3:5-diiodothyronine, thyroxine, thyroglobulins of human origin, iodocasein, and plastein from iodocasein.

2. Differences were revealed in the X-ray diffraction patterns of the various iodinated amino-acids, indicating some kind of structural rearrangement in the course of iodination.

3. The X-ray diffraction patterns of thyroglobulins of varying thyroxine content and of iodocasein are identical with the X-ray diffraction patterns of the corresponding iodine-free proteins. In artificial mixtures a content of 2% thyroxine seems to be the lower limit for detection by X-ray diffraction methods. Diiodotyrosine admixtures corresponding to the iodine content of iodinated casein are plainly visible in the X-ray diffraction pattern of the casein-diiodotyrosine mixtures.

4. Plastein made from iodocasein shows X-ray diffraction patterns different from those of the latter substance.

REFERENCES

Cavett, J. W., Rice, C. O. & McClendon, J. F. [1935]. *J. biol. Chem.* **110**, 673.
Cavett, J. W. & Seljeskog S. R. [1933]. *J. biol. Chem.* **100** xxci.
Clark G. L. [1940]. Applied X-Rays. New York and London: McGraw Hill Book Co.
Flosdorf, E. W. [1941]. *Science*, **93**, 157.
Hanawalt, D. J., Rinn, H. N. & Frevel, L. K. [1938]. *Industr. Engng Chem.* (Anal ed.) **10**, 457.
Harington, C. R. & Barger, G. [1927]. *Biochem. J.* **21**, 169.
Harington, C. R. & McCartney, W. [1927]. *Biochem. J.* **21**, 852.
Harington, C. R. & Pitt Rivers, R. V. [1939]. *Nature, Lond.*, **144**, 205.
Heidelberger, M. & Pedersen, K. O. [1935]. *J. gen. Physiol.* **19**, 95.
Katz, J. R. [1934]. Die Roentgen spektrographie als Untersuchungsmethode. *Handb. biol. Arbeitsmethoden*, ed. by E. Abderhalden, II/3/6.
Ludwig, W. & Mützenbecher, P. v. [1939]. *Hoppe-Seyl. Z.* **258**, 195.
Salter, W. T. [1935]. *Amer. J. Physiol.* **113**, 114.
—— [1940]. The Endocrine Function of Iodine, p. 60. Harvard Univ. Press.
Spiegel-Adolf, M. & Henny, G. C. [1939]. *J. Amer. chem. Soc.* **61**, 2178.
—— —— [1941]. *J. Phys. Chem.* **45**, 931.
—— —— [1942]. *J. Phys. Chem.* **46**, 581.
Spiegel-Adolf, M. & Peckham, R. H. [1940]. *Industr. Engng Chem.* (Anal. ed.), **12**, 182.
Wasteneys, H. & Borsook, H. [1924–5]. *J. biol. Chem.* **62**, 15.

98. Studies in the Biochemistry of Micro-organisms

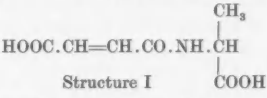
71. Fumaryl-*dl*-alanine (Fumaromono-*dl*-alanide), a Metabolic Product of *Penicillium resticulosum* sp.nov.

By **John Howard Birkinshaw**, **Harold Raistrick** and **George Smith**, *From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London*

(*Received 12 August 1942*)

A systematic investigation of the production of antibacterial substances by moulds is being carried out in this laboratory and has already led to the identification of a number of mould metabolic products and related substances which have striking antibacterial properties [Raistrick & Smith, 1941; Oxford, Raistrick & Smith, 1942; Oxford, 1942 *a*, *b*; Oxford & Raistrick, 1942]. In the course of this investigation it was noticed that a new species of *Penicillium*, *P. resticulosum* sp.nov., when grown on Czapek-Dox 5% glucose solution, gives metabolism solutions which suppress completely the growth of *Staphylococcus aureus* in bullock's heart broth containing 2% of glucose at dilutions of 1 : 320.

This antibacterial substance is very sensitive towards acids so that metabolism solutions from *P. resticulosum*, showing full activity at dilutions of 1 : 320, become entirely inactive on keeping overnight at *p*H 2·0 or below. Ether extraction of such inactivated metabolism solutions led to the isolation, in considerable yield, of a new mould metabolic product of Structure I. This substance, which may be regarded as a simple peptide of fumaric acid and *dl*-alanine, has, for convenience, been named *fumaryl*-dl-*alanine* in accordance with the ordinary peptide nomenclature although this name is not strictly correct, a more accurate designation being *fumaromono*-dl-*alanide*.



Structure I

The intriguing possibility therefore arose that this compound forms an integral part of the molecule of the antibacterial substance produced by this mould. Unfortunately such evidence as we have been able to adduce up to the present, while not absolutely conclusive, tends to disprove this possibility. Fumaryl-*dl*-alanine is a strongly acidic substance and hence cannot be extracted with ether from active metabolism solutions (*p*H 4·1) without prior acidification to *p*H 2 or below, a proceeding which itself inactivates the antibacterial substance. However, we have found that the antibacterial substance is precipitated in a crude but active form by addition of acetone to the mould metabolism solution. This active precipitate is completely inactivated on treatment with cold *N*/10 HCl but no fumaryl-*dl*-alanine was obtained on ether extraction of the inactivated solution.

From what is known at present of the properties of the antibacterial substance from *P. resticulosum* it is clear that it is not penicillin.

The molecular constitution of the new mould metabolic product as fumaryl-*dl*-alanine was established as follows. The substance crystallizes in colourless needles, m.p. 229° (decomp.) having the empirical formula $C_7H_9O_5N$ and titrates as a dibasic acid. It contains no amino-acid nitrogen liberated on treatment with nitrous acid. Hydrolysis by boiling with $2N$ H_2SO_4 for 17 hr. converts all the nitrogen into amino-acid nitrogen and the products of hydrolysis, isolated in approximately equimolecular proportions, are fumaric acid and *dl*-alanine.

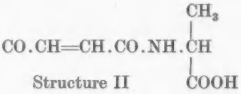
$$C_7H_9O_5N + H_2O \rightarrow HOOC.CH=CH.COOH + CH_3.CH(NH_2).COOH.$$

The fumaric acid was characterized by analysis, titration and mixed m.p. with an authentic specimen. Its identity was confirmed by the preparation of its characteristic reaction product with diazomethane, i.e. dimethyl-4:5-pyrazolinedicarboxylate [v. Pechmann, 1894], m.p. and mixed m.p. with an authentic specimen, 96–97°.

The *dl*-alanine was characterized by analysis and by comparison of the natural product and of its picrolonate with authentic synthetic *dl*-alanine and its picrolonate.

The mould metabolic product, on catalytic reduction with palladium-charcoal-hydrogen, gives *succinyl*-dl-*alanine*, colourless needles, m.p. 157°.

The molecular constitution was confirmed by synthesis. Synthetic *dl*-alanine (1 mol.) was treated in dry ether with fumaryl chloride (1 mol.), Cl.CO.CH=CH.CO.Cl. The resulting condensation product,



Structure II

on treatment with water, gave fumaryl-*dl*-alanine identical in its properties with the natural product.

We have been at some pains to establish beyond reasonable doubt the fact that the mould metabolic product is optically inactive and that the amino-acid hydrolysis product is indeed *dl*-alanine and not one of the optically active forms. This is a matter of some importance in view of the rare occurrence in nature of *dl*-amino-acids though, in this connexion, it may be significant that *dl*-alanine has been previously recorded as a fungal metabolic product. Winterstein, Reuter & Korolew [1913] isolated *dl*-alanine from an aqueous extract of the higher fungus *Boletus edulis*. Our evidence is the following: (*a*) A solution of the natural product in ethanol, equivalent to 6·63 g. in 100 g. of solvent gave no measurable rotation in a 0·5 dm. tube. (*b*) A 5 % solution in 2*N* H_2SO_4 after complete hydrolysis gave no measurable rotation in a 4 dm. tube. (*c*) An aqueous solution of the hydrochloride of the alanine isolated from the hydrolysed metabolic product (*c* = approx. 10 %) gave no measurable rotation in a 0·5 dm. tube. Fischer [1906] gives the rotation of *d*-alanine hydrochloride as $[\alpha]_D^{20°} = +10{\cdot}4°$ (*c* = 10 % in water).

Experimental

Culture

The culture used throughout this work was *Penicillium resticulosum* sp.nov., L.S.H.T.M. catalogue no. P 47. Since this species is a new one and has not been described previously a full diagnosis is given.

Diagnosis

Penicillium resticulosum sp.nov. Coloniis in agaro Czapekii late crescentibus, raris, primo albis, dein subglaucis cum marginibus funiculosis latissimis, denique aequaliter griseo-viridibus, floccosis et funiculosis, late zonatis in cultis senibus; guttulis crebris, minutis, subvinosis; reverso celeriter castaneo in media parte, succineo in margine, tarde subzonato; conidiophoris longis, funiculosis non vere fasciculatis, glabris, 3–4μ diam.; penicillis compactis, bi- trive-verticillatis, plerumque cum axe principale, metulas sterigmatesque gerente, et uno duobusve ramis secundariis confertis ex nodis inferioribus, ad 100μ long.; metulis 14–19 × 3·3–5μ, subclavatis aut cum apicibus inflatis; sterigmatibus 9–10 × 2·2–3μ; conidiis primo citriformibus catenis oidiorum similibus, deinde subglobosis, glabris, 3–4 × 2·8–3·2μ, in catenis primo parallelis demum implicatis.

Coloniis in musto ex hordeo cum agaro in tenebris diu sterilibus, in luce conidia celeriter gerentibus.

Fungus alium cultum contaminans inventus.

Colonies on Czapek agar spreading broadly, tenuous, at first white then pale bluish green with very broad funiculose margin, becoming fairly dark grey-green all over, floccose funiculose, especially in outer areas, broadly and indistinctly zoned in old cultures; drops numerous during the growing period, small, pinkish; reverse quickly reddish brown to chestnut brown, shading to dull yellow near the margin; conidiophores long, mostly loosely aggregated in ropes rather than in true fascicles, smooth, 3–4μ in diam.; penicilli very compact, 2- to 3-verticillate, often with main axis bearing a terminal verticil of metulae and sterigmata and one or two appressed secondary branches bearing sterigmata at approximately the same level as the main axis, up to 100μ in total length; metulae somewhat clavate or definitely swollen at the tip, 14–19 × 3–3·5μ (4·5μ at tip); sterigmata 9–10 × 2·2–3μ, widening upwards then tapering abruptly to fine points; conidia cut off as more or less citriform segments, often resembling chains of oidia, subglobose when ripe, smooth, 3–4 × 2·8–3·2μ; conidial chains at first roughly parallel, in old cultures becoming much tangled.

Cultures on wort agar remain sterile for several weeks if kept in the dark but well established colonies may be induced to form abundant spores by exposure for a few hours to diffuse light.

Related to *P. terrestre* Jensen and *P. solitum* Westling, but differing from both in texture of colonies and in colour.

Found as a culture contaminant in the laboratory. The name chosen for the new species is descriptive of the colony texture. (Latin *resticula* = a thin rope or cord.)

Cultural conditions

The culture medium used was a Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1000 ml.; initial *p*H, 4·3. This medium was distributed in 350 ml. amounts in each of 100 one-litre conical flasks plugged with cotton wool. Fifty of the flasks were sterilized without further treatment (batch I); 1 ml. *N* NaOH was added to each of the remaining 50 flasks before sterilization, thus raising the initial *p*H to 6·0 (batch II). After sterilization, all flasks were sown with a spore suspension of *P. resticulosum*, P 47, prepared from cultures grown for 2 weeks on beer-wort agar and were then incubated in the dark at 24°.

The mould quickly formed a substantial felt, white at first, gradually developing patches of tan and purplish brown on a dirty white background, with edges showing streaks of brownish purple and reverse of felt yellowish tan. Sporing was spasmodic and was usually delayed until the cultures were about 3 weeks old, when a proportion of the flasks began to show floccose patches of greyish blue-green, particularly evident round the edges of the felt. The culture medium rapidly became dull yellow, darkening only a little on continued incubation. Filtered metabolism solutions, however, rapidly darkened to brownish orange on standing. There was no obvious difference in appearance between batches I and II.

Isolation and purification of fumaryl-dl-*alanine*

At the end of the incubation period the metabolism solution from batch I was filtered from the mycelium which was washed well with distilled water. The filtrate and washings were evaporated in vacuo to about 400 ml. and the concentrate was acidified by the addition of 5*N* HCl (100 ml.). The acidified liquid was shaken with ether (1 litre), the acid extracted by the ether then being transferred to saturated aqueous $NaHCO_3$ (20 ml.). The ether was now used again, without further treatment, to re-extract the acidified metabolism solution, the whole process being repeated 10 times. After the first two extractions the amount of $NaHCO_3$ used was reduced to 10 ml. each time. The combined $NaHCO_3$ extracts were acidified with HCl and subjected to continuous extraction with

ether. The solid crystallizing from the ether each day was removed, the extraction being continued for four working days. A total of 2·91 g. of crystals, m.p. 226°, was thus obtained. Evaporation of the ether mother liquors yielded a further 0·42 g. of slightly less pure crystalline material and finally an oily residue, wt. 4·05 g. Total yield of crude fumaryl-*dl*-alanine from 50 flasks of batch I = 3·33 g.

Fifty flasks of batch II, on similar treatment, gave 3·96 g. of crude fumaryl-*dl*-alanine and an oily residue.

Experimental details of batches I and II are summarized in Table 1.

Table 1

Batch	Incubation period days	Initial *p*H	Final *p*H	Residual glucose by polarimeter %	Yield of crude product g.
I	19	4·3	4·1	0·81	3·33
II	20	6·0	4·1	0·80	3·96

The crude crystalline fumaryl-*dl*-alanine, m.p. 226°, obtained from both batches was already fairly pure. On recrystallization from glacial acetic acid with the addition of a little decolorizing carbon the m.p. was raised to 228°. Finally, recrystallization from water gave a product with a constant m.p. of 229° (decomp.).

General properties of fumaryl-dl-*alanine*

The substance crystallizes in colourless needles, m.p. 229° (decomp.). It is readily soluble in ethanol, hot water (cold water, 2–3 %), hot glacial acetic acid (cold, about 1 %), slightly soluble in ether, insoluble in light petroleum. It is a strong acid and is soluble in aqueous $NaHCO_3$ or Na_2CO_3 with the liberation of CO_2. It gives a strong fluorescein reaction when tested according to Feigl [1939, p. 299] for 1:2-dicarboxylic acids, probably due to hydrolysis to fumaric acid and alanine during the heating with conc. H_2SO_4 and resorcinol.

It is optically inactive. 0·0512 g. dissolved in 0·7718 g. of ethanol ($\equiv$ 6·63 g. in 100 g. of ethanol) gave no rotation in a 0·5 dm. tube in the mercury green light, $\lambda = 5461$. (Found: C, 44·80; H, 4·98; N, 7·42 %; CH_3O, nil; equiv. by titration, 94·5. $C_7H_9O_5N$ requires C, 44·92; H, 4·85; N, 7·49 %; equiv. titrating as a dibasic acid, 93·6.)

Derivatives of fumaryl-dl-*alanine*

(*a*) *Reaction product with diazomethane.* The metabolic product (0·1 g.) suspended in ether (5 ml.) was treated with an excess of diazomethane in ether. Brisk evolution of nitrogen occurred and the metabolic product dissolved. Later the reaction product separated in crystalline form and was collected. Wt. 0·088 g.; m.p. 162–165°. It was purified by recrystallization from ether followed by sublimation in a high vacuum at 115°. Colourless micro-crystals which on heating sintered above 160°, melted at 164–167° when tiny bubbles were evolved and the liquid cleared at 169°. It reset at 152° to a pasty crystalline mass which remelted at 169°, again evolving bubbles. There was no appreciable darkening. (Found: C, 46·96, 46·95; H, 5·81, 5·86; N, 15·8; CH_3O, 23·3 %. $C_{10}H_{15}O_5N_3$ requires C, 46·69; H, 5·88; N, 16·3; $2CH_3O$, 24·1 %.) This reaction product is obviously derived by the methylation of two COOH groups and the addition of CH_2N_2, in a manner analogous to the reaction of fumaric acid with diazomethane, see p. 833.

(*b*) *Catalytic reduction product. Succinyl*-dl-*alanine.* The metabolic product (0·43 g.) dissolved in ethanol (12 ml.) was reduced with a palladium-charcoal catalyst (from 0·1 g. of palladium chloride) in an atmosphere of hydrogen. Observed uptake of hydrogen, 49·8 ml. (corr.); calc. for uptake of 2H per mol., 51·5 ml. The catalyst was separated by filtration and on removal of the solvent in vacuo a colourless crystalline solid remained (wt. 0·44 g.; m.p. 145° to a cloudy melt clearing at 150°). The crude product was crystal-

lized from glacial acetic acid and *succinyl*-dl-*alanine* was thus obtained as colourless needles, m.p. 157°, which are readily soluble in water and ethanol, slightly soluble in ether and insoluble in light petroleum. (Found: C, 44·69; H, 5·84; N, 7·45 %. $C_7H_{11}O_5N$ requires C, 44·44; H, 5·86; N, 7·40 %.)

Acid hydrolysis of fumaryl-dl-*alanine*

A mixture of the metabolic product (2·037 g.) and 2*N* H_2SO_4 (40 ml.) was boiled under reflux. The metabolic product quickly dissolved. At intervals the solution was cooled and 0·2 ml. was withdrawn for an amino-acid nitrogen estimation by the Van Slyke method. The amino-acid nitrogen found after various intervals was as follows: 0 hr., nil; 2 hr., 0·219 mg.; 5 hr., 0·560 mg.; 9½ hr., 0·643 mg.; 13½ hr., 0·707 mg.; 17 hr., 0·753 mg.; calc. for conversion of all nitrogen present to amino-acid nitrogen, 0·763 mg. After 17 hr. the hydrolysis was stopped and, on cooling, a colourless crystalline substance (A) separated. The clear supernatant liquid (B) showed no rotation in a 4 dm. tube.

Isolation and characterization of fumaric acid. The colourless crystalline substance (A) was separated by filtration, washed with water and dried. Wt., 0·87 g.; m.p. (in a sealed tube) 286°, alone or in admixture with authentic fumaric acid. The supernatant liquid (B) and washings, on continuous extraction with ether, yielded 0·30 g. of slightly impure fumaric acid. Total yield, 1·17 g.; calc. 1·26 g. The fumaric acid was purified for analysis by sublimation in a high vacuum. (Found: C, 41·43; H, 3·52 %; equiv. by titration, 58·3. $C_4H_4O_4$ requires C, 41·39; H, 3·47 %; equiv. titrating as a dibasic acid, 58·0.)

The identity of the fumaric acid was confirmed by conversion into the characteristic dimethyl-4:5-pyrazolinedicarboxylate [v. Pechmann, 1894]. The acid (0·1 g.) was suspended in ether (15 ml.) and treated with an excess of diazomethane in ether. When the vigorous reaction was complete, part of the ether was removed. On standing overnight the solution deposited a lustrous, colourless mass of plates, m.p. 94–95°, raised to 96–97° by sublimation in a high vacuum and unchanged on admixture with dimethyl-4:5-pyrazolinedicarboxylate prepared by the same method from authentic fumaric acid. (Found: C, 45·35; H, 5·56; N, 15·4; CH_3O, 33·2 %. $C_7H_{10}O_4N_2$ requires C, 45·16; H, 5·41; N, 15·05; $2CH_3O$, 33·3 %.)

Isolation and characterization of dl-*alanine.* The supernatant liquid (B) and washings, after ether extraction, were quantitatively freed from H_2SO_4 by the addition of $Ba(OH)_2$. The filtrate was concentrated in vacuo and finally dried over conc. H_2SO_4 to give a colourless crystalline residue. Wt., 0·67 g.; calc., 0·97 g.; m.p. 276°. (Note that the calculated theoretical yields of fumaric acid and alanine are not corrected for the amounts of hydrolysis solution used for amino-acid nitrogen estimations.) Recrystallization of the crude product from hot water (10 ml.)+hot ethanol (40 ml.) yielded 0·38 g. of rods or blunt-ended needles, m.p. 290° (decomp.) and a second crop, wt., 0·12 g., m.p. 280° (decomp.). On sublimation in a high vacuum at 180°, micro-crystals (needles) were obtained, m.p. 298° (decomp.) alone or in admixture with sublimed, synthetic, *dl*-alanine. (Found: C, 40·65; H, 8·00; N, 15·5 %. $C_3H_7O_2N$ requires C, 40·43; H, 7·92; N, 15·7 %.)

The amino-acid had no optical rotation. 0·050 g., dissolved in 2*N* HCl (0·28 ml.) and diluted with water to a total weight of 0·5362 g., showed no rotation in a 0·5 dm. tube. Mean of several readings (*a*) mercury green light, $\lambda = 5461$, $-0·001°$; (*b*) mercury yellow light, $\lambda = 5790$, $-0·002°$.

The identity of the *dl*-alanine from the mould metabolic product was confirmed by preparation of the picrolonate. The amino-acid (0·1 g.) and picrolonic acid (0·3 g.) were dissolved in hot water (3·5 ml.), filtered and allowed to cool. The yellow needles thus obtained were collected and washed with a little water. Wt., 0·32 g.; m.p. 214° (decomp.). After grinding the dry crystals with ether the m.p. was raised to 216–217° (decomp.) alone or in admixture with the picrolonate of synthetic *dl*-alanine. (Found: C, 43·75; H, 4·50; N, 19·1 %. $C_{13}H_{15}O_7N_5$ requires C, 44·19; H, 4·28; N, 19·8 %.)

Synthesis of fumaryl-dl-*alanine.* (*Structure I*)

This synthesis was carried out following the method of Anschütz [1890] for the preparation of fumaranilic acid. Synthetic *dl*-alanine (0·5 g.) was covered with dry ether (5 ml.) and treated with 0·3 ml. (=1 mol.) of fumaryl chloride. The mixture was shaken occasionally. The ether soon became bright yellow in colour. After 6 hr. the ethereal solution was filtered from the insoluble matter (probably alanine hydrochloride) which was washed with dry ether. The solvent was removed and the solid residue was dried in a good vacuum over solid KOH to remove traces of fumaryl chloride. Wt., 0·41 g.; m.p. 194° (decomp.). On treatment with water (20 ml.) the condensation product (Structure II of introduction) slowly dissolved. The aqueous solution was subjected to continuous ether extraction and yielded a product (0·35 g.) which was refluxed with ether (15 ml.). The residual crystalline mass (0·20 g.) was recrystallized from glacial acetic acid (3 ml.) and fumaryl-*dl*-alanine (0·12 g.) was thus obtained as colourless needles, m.p. 229° (decomp.), not depressed on admixture with the mould metabolic product, m.p. 229° (decomp.). (Found: C, 44·70; H, 4·97; N, 7·0 %. $C_7H_9O_5N$ requires C, 44·92; H, 4·85; N, 7·5 %.)

Antibacterial properties of the metabolism solution of P. resticulosum

100 flasks of Czapek-Dox solution (initial *p*H 4·3) were prepared, sown with *P. resticulosum* and incubated exactly as described for batch I (see p. 831). They were tested at intervals for antibacterial activity by the method used here originally by Clutterbuck, Lovell & Raistrick [1932] for the assay of solutions of penicillin and later by Raistrick & Smith [1941] and by Oxford [1942 *a*] for the assay of other mould metabolic products showing antibacterial activity, e.g. citrinin and penicillic acid.

Serial dilutions of the filtered mould metabolism solution were made from 1 : 10 to 1 : 5120 in 5 ml. amounts of sterile broth (bullock's heart broth containing 0·5 % NaCl, 1 % Witte's peptone and 2 % glucose; final *p*H, after sterilizing, 7·2). Each tube was inoculated with one standard loopful of a 24 hr. culture of *Staphylococcus aureus*, L.S.H.T.M. strain, in the same medium. The inoculated tubes were incubated at 37° and the results were read after 24 hr. The results obtained are summarized in Table 2.

Table 2

Incubation period days	Number of flasks	Glucose by polarimeter %	*p*H	Inhibition against *Staphylococcus aureus*	
				Complete	Partial
14	4	1·53	3·8	1 : 320	—
15	50	1·26	3·85	1 : 320	1 : 2560
21	1	0·53	4·0	1 : 320	1 : 2560
23	45	0·46	4·3	1 : 200	1 : 400

The figures in the second column give the number of flasks which were taken off and of which the contents were mixed before filtration and assay.

Similar tests carried out on batch I (initial *p*H 4·3; 19 days' incubation) and batch II (initial *p*H 6·0; 20 days' incubation) (see Table 1) gave complete inhibition of growth in both cases at dilutions of 1 : 320 after 24 hr. incubation of the inoculated broth cultures at 37°.

The marked susceptibility of this antibacterial substance to acid was demonstrated as follows. 10 ml. of the average sample from 50 flasks taken off after 15 days' incubation (see Table 2) were acidified with 1 ml. *N* HCl, held at room temperature overnight and then neutralized with 1 ml. *N* NaOH. At the same time a control was set up in which 2 ml. *N* NaCl were added to another 10 ml. of the same sample after standing overnight. Antibacterial tests showed full growth of *Staphylococcus aureus* at all dilutions of the acidified sample but complete inhibition of growth at 1 : 320 and partial inhibition at 1 : 1280 in the control.

Isolation and general properties of the crude antibacterial substance

The antibacterial substance present in the metabolism solution of *P. resticulosum* has not yet been obtained pure. Hence only the results of preliminary experiments will be given here, fuller details being reserved for a later communication.

The filtered metabolism solution from 100 flask cultures grown on Czapek-Dox solution (initial *p*H 4·3) and giving total inhibition of the growth of *Staphylococcus aureus* at a dilution of 1 : 80 was evaporated in vacuo (bath temperature 40–45°) to about 1/7 of its volume. During this process about one-half of the original antibacterial activity was lost. An equal volume of acetone was added to the concentrate and after standing overnight at 0° the precipitate was separated by centrifuging. The supernatant aqueous acetone solution, after removal of the acetone, contained very little antibacterial substance. The acetone precipitate was extracted with cold distilled water (100 ml.), re-centrifuged and re-extracted with 100 ml. of water. The combined aqueous extracts (200 ml.) were treated with acetone (400 ml.) and the resulting precipitate was separated by centrifuging and dried in vacuo. Wt. 3·87 g.

The crude antibacterial substance thus obtained has the following properties. It resembles a pale brownish-yellow resin which can be easily powdered and it is readily soluble in water. It contains nitrogen, 3·8 % of ash and gives a positive Molisch reaction. It completely inhibits the growth of *Staphylococcus aureus* in glucose broth at a concentration of 1 : 160,000 with partial inhibition of growth at 1 : 320,000. A 1 % solution in *N*/10 HCl is completely inactivated on standing overnight at room temperature. A solution of the substance (1 g.) in *N* HCl (50 ml.) yielded only a trace (about 5 mg.) of extract on prolonged extraction with ether. This extract could not be identified as fumaryl-*dl*-alanine. If the scission of fumaryl-*dl*-alanine from the antibacterial substance on treatment with dilute acid were in fact the cause of the loss of antibacterial activity its presence would almost certainly have been revealed by the above procedure (see p. 831).

SUMMARY

Penicillium resticulosum sp.nov., when grown on Czapek-Dox 5 % glucose solution, produces considerable quantities of the hitherto undescribed fumaryl-*dl*-alanine (fumaromono-*dl*-alanide); this substance has been synthesized. Metabolism solutions of *P. resticulosum* show considerable antibacterial activity and the antibacterial substance has been isolated in a crude form. Fumaryl-*dl*-alanine does not appear, however, to form an integral part of the molecule of the antibacterial substance.

We desire to thank Mr W. K. Anslow and Mr A. Bracken for much technical assistance during this investigation.

REFERENCES

Anschütz, R. [1890]. *Liebigs Ann.* **259**, 137.
Clutterbuck, P. W., Lovell, R. & Raistrick, H. [1932]. *Biochem. J.* **26**, 1907.
Feigl, F. [1939]. Qualitative Analysis by Spot Tests. 2nd ed. London: Elsevier Publishing Co., Ltd.
Fischer, E. [1906]. *Ber. dtsch. chem. Ges.* **39**, 453.
Oxford, A. E. [1942*a*]. *Chem. Ind.* **61**, 48.
—— [1942*b*]. *Chem. Ind.* **61**, 189.
Oxford, A. E. & Raistrick, H. [1942]. *Chem. Ind.* **61**, 128.
Oxford, A. E., Raistrick, H. & Smith, G. [1942]. *Chem. Ind.* **61**, 22.
v. Pechmann, H. [1894]. *Ber. dtsch. chem. Ges.* **27**, 1888.
Raistrick, H. & Smith, G. [1941]. *Chem. Ind.* **60**, 828.
Winterstein, E., Reuter, C. & Korolew, R. [1913]. *Landw. VersSta.* **79/80**, 541.

99. Metabolism of Sulphur. Cysteic Acid*

By **Grace Medes** and **Norman Floyd**, *From the Lankenau Hospital Research Institute, Philadelphia, Pa.*

(*Received 1 September 1942*)

The place of cysteic acid in metabolism has never been adequately determined. Earlier experiments [Schmidt & Clark, 1922; Andrews & Randall, 1935; White, Lewis & White, 1937; Medes, 1937] were designed to ascertain whether this substance is in the path of the oxidation of cystine to inorganic sulphate. In general it was concluded that while ingested cysteic acid may give rise to small amounts of inorganic sulphate, cysteic acid injected intravenously or subcutaneously does not contribute to sulphate formation. Hence, the conclusion was reached that cysteic acid cannot be an intermediate in the oxidation of cystine to inorganic sulphate.

That cysteic acid is a physiologically occurring product has been shown more recently by Bernheim & Bernheim [1939] and Medes [1939] who extracted an enzyme from liver which oxidizes the sulphur of cysteine to the sulphonic acid stage. However, no hepatic enzyme which acts on cysteic acid has been found.

Two possible clues to its fate have been suggested. (*a*) Virtue & Doster-Virtue [1939] showed that cysteic acid ingested with cholic acid increases the output of taurocholic acid in the bile of dogs, but the mechanism for its conversion into taurine has not been discovered. (*b*) Schmidt & Clark [1922] found that following oral administration of cysteic acid without accompanying cholic acid a deaminated product appeared in the urine. Such a product could not have been taurine. Very suggestive in connexion with this finding of Schmidt & Clark, is the report of Cohen [1940] that cysteic acid may participate in the transamination activities of muscle tissue according to the formulas: α-ketoglutaric acid + *l*-cysteic acid $\rightarrow$ *l*-glutamic acid + β-sulphopyruvic acid, and oxaloacetic acid + *l*-cysteic acid $\rightarrow$ *l*-aspartic acid + β-sulphopyruvic acid. If the β-sulphopyruvic acid is excreted unchanged the appearance of a deaminated product of cysteic acid would be accounted for; but since this would seem to be a very unstable substance, further investigation was advisable to determine whether it is excreted entirely in this form or whether part of it slowly gives rise in the tissues to inorganic sulphate. Medes [1937] had observed that there was a rise in the sulphate elimination beginning about 20 hr. after ingestion of 3·64 g. of cysteic acid and that this elevated level was continued for approximately 24 hr. The rise in sulphate excretion might be explained on the basis of intestinal putrefaction, but it might also be due to some delayed mechanism in the tissues, such as transamination to sulphopyruvic acid and subsequent oxidation of the sulphur to sulphate.

Metabolism of cysteic acid following ingestion of small amounts over a 42 hr. period

It was thought that if transamination of cysteic acid to sulphopyruvic acid occurred in the muscle, followed by sulphate formation, the process would probably be slow and the amount oxidized to sulphate would be limited by the rate of transamination. It is possible that this latter process could not be speeded up sufficiently to care for unphysiologically large amounts of cysteic acid (up to 15 g.) administered at one time in some of the experiments previously reported. In the following experiment the amount ingested was cut as low as was consistent with the sensitiveness of the several tests employed and was administered in a series of small doses over a prolonged period.

* Aided by a grant from the McNeil Laboratories, Inc., Philadelphia, Pa.

The subject was the same as was used for previous observations [Medes, 1937]. 1·848 g. of cysteic acid were dissolved in such a volume of water that 2 ml. contained 22 mg. A 2 ml. portion was taken by mouth every ½ hr. for 42 hr. except for a 6 hr. period during the night when double the dose was consumed hourly. 1·056 g. were therefore ingested during the first 24 hr. and 792 mg. during the following 18 hr. The subject was on the same low protein, low sulphur diet as was employed in previous experiments [Medes, 1937]. Urine was collected in 6 hr. periods and analysed for total and neutral S, ethereal and inorganic sulphates and total N. Fig. 1 shows the results for the various periods.

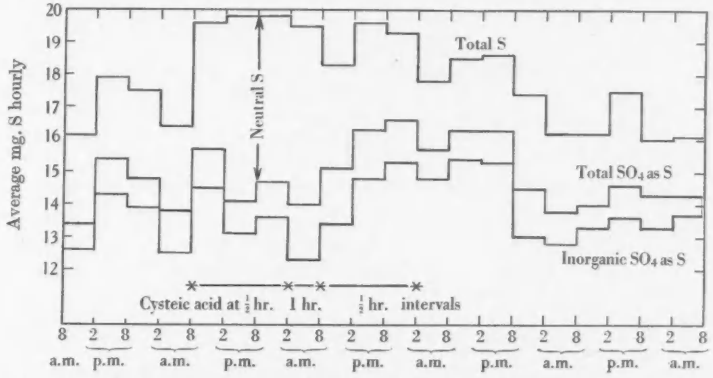


Fig. 1. Excretion of sulphur during an experiment in which 22 mg. of cysteic acid were ingested every half-hour for 42 hours, except during 6 hours, in which 44 mg. per hour were taken.

It may be seen that excretion of extra S begins immediately after ingestion of the cysteic acid and for 30 hr. is entirely in the neutral S class. Afterwards neutral S falls to the normal value and sulphate-S begins to increase. The average level of total S was 16·9 mg./hr. before the first experimental period, was maintained at a fairly even level of 19·5 mg./hr. during the ingestion period and immediately fell to an average of 18·8 mg./hr. during the following 6 hr., and to 17·2 mg. during the next 24 hr. Total sulphate, on the other hand, averaged 14·4 mg./hr. in the pre-ingestion period, 14·6 mg./hr. during the first 24 hr. of the ingestion period, then rose to an average of 16·1 mg./hr. during the subsequent 36 hr.; 32% of the extra S was recovered, of which 16·1 % was determined as neutral S and 15·9 % as sulphate-S.

Although the delayed, slow rise in sulphate occurred as in previous experiments, the total amount recovered as sulphate was not greater than when a single large dose was administered and did not constitute a greater percentage of the ingested cysteic acid. This evidence, therefore, while not conclusive, seemed to indicate either that sulphate formation following transamination did not take place, or that the normal amount could not be increased even by prolonged ingestion of small amounts of cysteic acid, and hence the process, if it occurred, could not be demonstrated by an experiment of this type.

Metabolism of cysteic acid in muscle

A second approach to the problem was through testing for sulphate formation by muscle and liver tissue in the presence of sulphopyruvic acid; the experiments were conducted as follows. Transaminase was prepared as described by Cohen [1940] and incubated in the presence of cysteic acid and oxaloacetic or α-ketoglutaric acid* for 1 hr.

* The authors are indebted to Dr P. P. Cohen for generously supplying α-ketoglutaric and oxaloacetic acids.

in 0·07 *M* phosphate buffer at *p*H 7·7, after which part of the incubated mixture was removed to test for activity of the transaminase. These tests were carried out in the Warburg apparatus with chloramine-T as described by Cohen. In two solutions tested, about 15 and 18 % transamination was found to have taken place.

To the remainder of the incubated enzyme and sulphopyruvic acid, was added muscle or liver tissue freshly ground in a Latapie mincer. A sample of the solution was removed immediately for sulphate estimation and the remainder incubated for 2 hr. in an atmosphere of oxygen. A second sample was then removed for sulphate estimation. A control tube containing all the components except substrate and keto-acid was treated similarly. Sulphate determinations were carried out by the method described by Medes & Floyd [1942]. In two experiments in which oxaloacetic acid was employed with cysteic acid as substrate in the transamination procedure and in three experiments in which α-ketoglutaric acid was used with cysteic acid, no sulphate production from sulphopyruvic acid by either liver or muscle tissue could be detected.

Tests for sulphopyruvic acid in urine following cysteic acid ingestion

The possibility that sulphopyruvic acid resulting from deamination of cysteic acid may be the form in which this S is excreted was the object of the next experiment. The same general procedure was employed as in the previous feeding experiments, with the subject on a constant diet of somewhat higher S content, beginning 6 days before the administration of cysteic acid. On the seventh day 4·488 g. of cysteic acid monohydrate (24 m.mol.) neutralized with NaOH were ingested in three portions at hourly intervals. The 24 hr. samples of urine were analysed for total S, total and inorganic sulphate and amino-N. Since it was hoped to detect the presence of sulphopyruvic acid in case cysteic acid was excreted in this form, keto-acids were estimated by the colorimetric procedure of Sealock [1941] with pyruvic acid as standard. Values are expressed in terms of pyruvic acid equivalents.

The results are given in Table 1. Total S rose from an average of 17·6 m.mol. daily to 27·5 on the first day after ingestion (day 7), to 21·8 on the second day, and returned to the normal value, 17·6 m.mol. on the third day. This represented a recovery of 58·1 % of the extra S ingested.

Table 1

Fate of ingested and injected cysteic acid by a normal human subject, wt. 60 kg.

Method of admin.	Day of exp.	Vol. ml.	Sulphur in m.mol. Total S	Neutral S	Total SO_4	Ethereal SO_4	Inorg. SO_4	Ketone m.mol.	Amino-N m.mol.
Ingested	1	775	18·56	2·21	16·35	0·89	15·46	0·41	4·32
	2	950	18·31	1·98	16·33	0·80	15·53	0·43	5·12
	3	670	17·66	2·10	15·56	0·82	14·74	0·39	4·27
	4	965	18·49	2·09	16·40	0·87	15·63	0·42	4·75
	5	1127	16·03	1·98	14·05	0·72	13·33	0·36	1·94
	6*	945	17·54	2·06	15·48	0·92	14·56	0·39	4·17
	Av.		17·74	2·07	15·69	0·84	14·88		
	7	735	27·58	2·06	25·52	3·69	21·83	0·47	3·34
	8	630	21·86	3·59	18·27	2·51	15·76	0·41	3·78
	9	950	17·68	2·54	15·14	0·83	14·31	0·40	4·09
Injected	3	680	10·66	1·92	8·74	0·98	7·76	0·56	2·96
	4†	685	10·10	2·02	8·08	0·96	7·14	0·39	2·50
	5	750	10·92	3·83	7·08	0·90	6·18	0·43	4·23
	6	600	9·23	2·15	7·08	0·92	6·16	0·43	3·51
	7	860	9·10	1·80	7·30	1·09	6·21	0·43	2·69

* 4·488 g. (24 m.mol.) neutralized cysteic acid were ingested in 3 portions at 8, 9 and 10 a.m.

† 1·318 g. cysteic acid (=7·5 m.mol.) neutralized with 19·42 ml. 0·3860*N* NaOH injected at 9.15 a.m. Same amount injected at 10 a.m.

In this experiment, in contrast to the earlier one, sulphate-S rose early, attaining an elevation of 25·5 m.mol. on the first subsequent day, dropping to 18·3 m.mol. on the second day and reverting to the normal value on the third. This represents 51·7 % of the added S or 88·9 % of the recovered S.

The rise in neutral S was slight. There was no increase on the first day after ingestion; on the second day the output rose from the average of 2·0 to 3·5 m.mol. On the third day it was still somewhat elevated, 2·5 m.mol., which corresponded to 8·3 % of the added S or 14·3 % of the recovered S. There was no increase in amino-N, and keto groups also remained at the control level. Hence sulphopyruvic acid could not have been present in more than traces among the excreted products.

Test for sulphopyruvic acid in urine following injection of cysteic acid

On account of the large amount of sulphate excreted and the complication of its possible formation from the action of intestinal flora, the experiment was repeated with cysteic acid injected intravenously. 15 m.mol. (2·636 g.) were administered in two portions with an interval of an hour. The results are given in Table 1. Total S was still falling towards the level, afterwards attained on the fourth day, of 9·2 m.mol. Considering any above this as extra S derived from the injected cysteic acid, 1·6 m.mol. of extra S were excreted during the 24 hr. following administration, or 11·3 % of that injected. It was all in the form of organic S. Ketones did not rise, but amino-N increased by 1·7 m.mol., a value approximately equal to the increase in neutral S, or 1·8 m.mol. The compound excreted could have been either taurine or unchanged cysteic acid. This evidence shows a direct relationship between the rise in the amino-N and the increase in the neutral S. Since unphysiological amounts of cysteic acid have been injected the results should, if transamination has taken place and if both end-products are excreted, show an increase in the keto group equivalent to the rise in the amino-N. Since the keto group is constant, evidently sulphopyruvic acid is not excreted. The strikingly different picture obtained upon injection of cysteic acid from that presented following ingestion, indicates either that bacterial destruction is very extensive or that the intestinal mucosa acts in some way upon absorbed cysteic acid with extensive disruption of the molecule.

To summarize: about 60 % of the S of cysteic acid ingested without accompanying cholic acid was excreted partly in the neutral S fraction of urine and partly as sulphate. There was no simultaneous rise of amino-N or of the keto group. When cysteic acid was injected intravenously only about 10 % of the S was recovered and was entirely in the organic S fraction. Urinary amino-N increased to about the same extent, while ketones remained constant.

Excretion in the bile

Although it has been amply shown that taurine, administered either orally [Schmidt, Adelung & Watson, 1918] or intravenously [Foster, Hooper & Whipple, 1919] without accompanying cholic acid, is excreted by way of the urine, it does not follow that cysteic acid behaves similarly since at this preliminary stage the union with cholic acid may be effected, resulting in a different disposition of the end-product. Virtue & Doster-Virtue [1939] found that cysteic acid injected into dogs appeared as taurine in the bile. Their dogs, however, were supplied with cholic acid and no studies were made by them in its absence. To ascertain whether that portion of the S of cysteic acid which does not appear in the urine may be excreted in some form in the bile, the following experiments were carried out on two patients who had been operated on for bile obstruction 4 days before initiating the tests. They were placed upon diets of controlled S content, and bile and urine were collected in 24 hr. periods. Results are given in Table 2. As may be seen, the volume of bile was normal. Urine was analysed for the usual S distribution, for amino-N before and after hydrolysis, and for bile acids. For the latter, the method of Gregory & Pascoe [1929] was employed. It was used unmodified in determining bile

Table 2

Excretion of S in the urine and bile of two bile fistula patients, following oral administration of cysteic acid. All values are expressed in m.mol.

				Sulphur				Amino-N				
Day	S in Diet	Vol. ml.	Creatinine	Inorg. SO_4	Total SO_4	Org. S	Total S	Before hydrol.	After hydrol.	Diff. (taurine + glycine)	Bile salts	Ketone
					Patient R. Urine*							
1	13·1	570	5·1	9·71	10·40	1·87	12·27	13·48	18·26	4·78	0·39	0·59
2†	12·6	765	4·8	9·13	9·67	1·36	11·03	15·69	19·90	4·21	0·39	0·61
3	13·4	1250	4·6	9·22	10·17	6·16	16·33	12·46	17·44	4·98	0·44	0·59
					Patient R. Bile*							
1	—	340	—	0·02	0·03	—	3·70	—	—	12·6	11·10	—
2†	—	407	—	0·03	0·05	—	3·45	—	—	15·2	15·10	—
3	—	320	—	0·34	0·47	—	3·54	—	—	9·3	9·72	—
					Patient W. Urine*							
1	5·0	1350	5·7	3·99	4·43	3·11	7·54	2·98	10·06	7·08	0·51	0·37
2‡	8·7	840	5·8	6·05	6·44	3·22	9·66	4·21	11·50	7·29	0·53	0·38
3	9·1	575	5·5	5·00	5·33	10·11	15·44	3·38	14·81	11·43	0·63	0·37
4	9·6	583	5·5	5·01	5·35	4·59	9·94	4·99	15·64	10·65	0·37	0·39
					Patient W. Bile*							
1	—	308	—	0·00	0·25	—	2·65	—	—	6·33	8·29	—
2‡	—	420	—	0·00	0·27	—	2·64	—	—	6·78	7·18	—
3	—	390	—	0·17	0·32	—	2·67	—	—	7·32	8·19	—
4	—	355	—	0·14	0·29	—	2·62	—	—	8·75	7·82	—

* Collections closed daily at 8.30 a.m.
† 12·8 m.mol. of cysteic acid administered at 8.30 a.m. and 12·8 m.mol. at 9.45 a.m.
‡ 10·6 m.mol. of cysteic acid administered at 8.30 a.m. and 10·6 m.mol. at 9·45 a.m.

acids in bile. For urine, the preliminary extraction with alcohol was omitted, the reaction being carried out directly on urine, employing as a blank a urinary sample similarly treated except for the omission of furfuraldehyde. Readings were taken in a photoelectric colorimeter with a 6200 Å. filter and estimations made from a standardized curve obtained with graded amounts of cholic acid. Patient R received 4·50 g. (25·6 m.mol.) of cysteic acid divided into two doses, at 8.30 and 9.45 a.m. on the third morning, and patient W received 4 g. (21·2 m.mol.) similarly administered on the third day. Table 2 gives the urinary and bile analyses for 2 days before ingestion of cysteic acid and for 1 and 2 days respectively, immediately after (collection of urine started at the time of administration).

As may be seen no significant change in S excretion by way of the bile was ascertainable. The neutral S fraction of urine increased, representing 19 and 33 % of the ingested S respectively. Amino-N (confirming the finding of Schmidt & Clark [1922]), except for a slight rise in patient W, was not elevated, and the keto reaction was unchanged. Thus only 19 and 33 % respectively was accounted for, and that, except for a small amount in W, could not have been cysteic acid, taurine or sulphopyruvic acid.

Action of various tissues on cysteic acid

Since ingested cysteic acid behaves differently from injected cysteic acid, an attempt was made to find out if some other tissue besides liver acts on it. Slices of liver, heart, intestines and kidney were incubated, under conditions similar to those previously employed. CO_2 and amino-N were determined before and after incubation. Parallel tests were conducted with and without addition of cysteic acid. The results are given in Table 3. It will be seen that liver, heart or muscle had no effect on cysteic acid. Kidney tissue acted on the carboxyl group, but had no effect on the N. Under the action of intestinal tissue, both amino-N and carboxyl completely disappeared.

Table 3

Effect of various tissues upon cysteic acid. Tissue slices were incubated in 0·02 *M* phosphate buffer at *p*H 7·4 which was 0·02 *M* with respect to cysteic acid. Controls were similarly incubated without cysteic acid. Figures represent m.mol./ml.

			CO_2			Amino-N		
Tissue	Cysteic acid added	Time (34°) hr.	In 0·002 *M* cysteic acid	Without cysteic acid	Difference	In 0·002 *M* cysteic acid	Without cysteic acid	Difference
Liver	0·02	0	0·0351	0·0150	0·0201	0·0227	0·0018	0·0209
		5	0·0335	0·0134	0·0201	0·0252	0·0045	0·0207
Heart	0·02	0	0·0335	0·0134	0·0201	0·0225	0·0020	0·0205
		5	0·0349	0·0148	0·0201	0·0258	0·0064	0·0194
Intestines	0·02	0	0·0335	0·0134	0·0201	0·0228	0·0027	0·0201
		5	0·0588	0·0620	0·0000	0·0619	0·0611	0·0008
Intestines	0·02	0	0·0335	0·0134	0·0201	0·0228	0·0027	0·0201
		5	0·0590	0·0600	0·0000	0·0426	0·0529	0·0000
Kidney	0·02	0	0·0351	0·0150	0·0201	0·0223	0·0021	0·0202
		5	0·0375	0·0219	0·0156	0·0248	0·0058	0·0190

To assure ourselves that this effect of intestinal enzymes was not due to the presence of intestinal bacteria, the experiment was repeated with the following additional procedures. After the regular incubation period, the medium surrounding the slices was strained through coarse filter paper to free it of tissue, and divided into two equal portions. To one was added cysteic acid so that the original concentration was restored. The other portion was passed through a Berkefeld filter to free it from any intestinal flora which might remain in the solution. To this portion, cysteic acid was also added in the same concentration. It was anticipated that if the disappearance of cysteic acid were due to the action of the intestinal flora, the substrate would be lost from the solution that had been strained through filter paper but be unaltered in the flask whose contents had passed through the Berkefeld filter. Results are given in Table 4. Actually, neither

Table 4

Action of intestinal tissue on cysteic acid. Concentration of cysteic acid was 2×10^{-2} m.equiv. per ml. of medium. Incubation was in 0·02 *M* phosphate buffer at *p*H 7·4 for 2 hr. Controls without cysteic acid were incubated similarly. Results are expressed in m.equiv. $\times 10^{-2}$ per ml. of medium.

		Carboxyl			Amino-N		
Period	Time hr.	With cysteic acid	Without cysteic acid	Difference	With cysteic acid	Without cysteic acid	Difference
Preliminary	0	0·0593	0·0388	0·0205	0·0247	0·0043	0·0204
	5	0·0587	0·0376	0·0211	0·0242	0·0046	0·0196
After straining through filter paper	0	0·0587	0·0376	0·0211	0·0239	0·0048	0·0196
	5	0·0523	0·0302	0·0223	0·0245	0·0051	0·0194
After passing through Berkefeld filter	0	0·0585	0·0377	0·0218	0·0246	0·0044	0·0201
	5	0·0558	0·0348	0·0210	0·0243	0·0041	0·0202

solution was changed, showing that the results were due to enzymes in the gut wall and not to the action of bacteria clinging to the intestinal surface. The fragments from the cysteic acid molecule were not identified. The possibility that the S fraction is ethyl sulphonic acid is now being investigated. Sulphate determinations were unsatisfactory owing to the relatively large amounts of sulphates in the tissue, but such assays as could be carried through indicated that sulphate was not formed.

Since kidney slices acted on the carboxyl group of cysteic acid without affecting the N, taurine apparently had been produced. An effort was made to extract the enzyme responsible. Table 5 gives the results of a series of experiments in which cysteic acid was incubated with kidney slices and kidney brei under both aerobic and anaerobic conditions.

Table 5

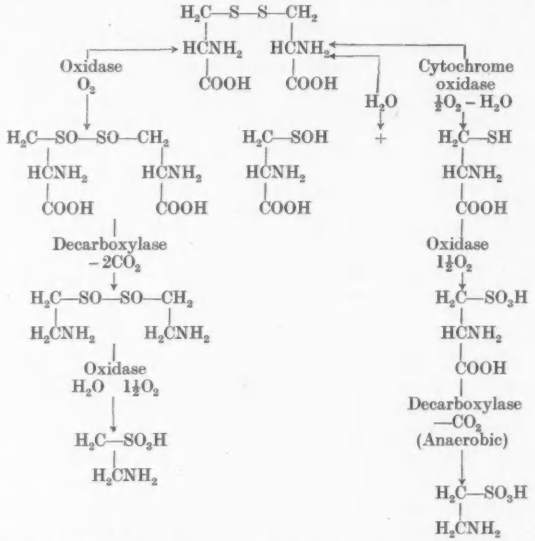
Decarboxylation and deamination of cysteic acid by liver and kidney of the rat. Substrate concentration was $2{\cdot}00 \times 10^{-2}$ m.equiv. per ml. of medium. Incubation was for 2 hr. in 0·02 *M* phosphate buffer at *p*H 7·4. Results expressed in m.equiv. $\times 10^{-2}$ per ml. of medium.

			Carboxyl		Amino-N	
Tissue	Condition	Substrate	Before	After	Before	After
Liver brei	Aerobic	Sodium cystinate	2·01	1·53	—	—
		Cysteic acid	2·03	2·04	—	—
		Leucine	2·03	1·82	—	—
		Histidine	2·03	0·92	—	—
Kidney slices	Aerobic	Cysteic acid	2·01	1·56	2·02	1·90
			2·01	1·53	2·00	1·98
			2·01	1·57	2·05	1·92
Kidney brei	Aerobic	Cysteic acid	2·01	1·42	2·05	1·21
			2·02	1·52	2·05	1·21
			2·01	1·52	1·92	0·00
Kidney brei	Anaerobic	Cysteic acid	2·02	1·35	2·05	2·07
			2·01	1·53	2·03	1·97

phosphate buffer 0·02 *M* at *p*H 7·4 was employed. It may be seen that while kidney slices convert cysteic acid into taurine, the brei, in the presence of oxygen, behaves apparently like the intestinal mucosa in that both amino and carboxyl groups disappear. In an atmosphere of N only the carboxyl radical is affected with resulting taurine formation.

Discussion

It has been shown [Medes & Floyd, 1942] that taurine may arise in the liver from cystine, which is first oxidized to cystine disulphoxide and then decarboxylated to the disulphoxide of cystamine. By a subsequent oxygen uptake, taurine is produced. The experiments recorded here show a second pathway for taurine formation through an anaerobic decarboxylation of cysteic acid. Blaschko [1942] isolated the enzyme from liver, while the present authors* found it in kidney. The results are summarized in the accompanying scheme.



* Reported by Medes & Floyd at the Meeting of the Society of Biological Chemists, April, 1942.

The various oxidative steps are enzymic, as are the decarboxylations. There are, therefore, three enzymes concerned in the production of taurine from cystine and two in the conversion of cysteine into the same end-product. In either case, cystine or cysteine could appear quantitatively as taurine. A question of fundamental importance is the interconvertibility of the cystine and cysteine systems.

Since sulphydryl may be oxidized quantitatively to disulphide by cytochrome oxidase, all of the cysteine could conceivably be converted into taurine through cystine and its disulphoxide. But starting with cystine, it could not all be transformed to taurine through cysteine. The latter is produced from cystine in the tissue by hydrolytic cleavage, and since cysteine sulphenic acid, formed as one of the products of hydrolysis, immediately dismutes to cysteine and cysteine sulphinic acid, theoretically only three-fourths of all cystine present could be converted into taurine through this channel. The remaining fourth, present as sulphinic acid, would be oxidized to inorganic sulphate. The sulphate portion might however be increased to include the entire product, if conditions in the tissues should be such that cytochrome oxidase was able to shunt all the cysteine back to the general reservoir, cystine, before the enzyme converting it into taurine could act.

At what stage do these reactions become irreversible? This question is answered better by feeding experiments which test the availability of the various end-products or of their metabolic intermediates for growth-promoting properties. Taurine cannot replace cystine in the diet [Lewis & Lewis, 1925; Beard, 1926] and hence it apparently cannot be converted by the body into cystine. This conclusion may also be drawn from the fact that taurine does not contribute to sulphate formation [Schmidt *et al.* 1918]. Cysteic acid does not increase urinary sulphate [White *et al.* 1937], hence its biological formation appears to be irreversible. Bennett [1937] reported that cystine disulphoxide is available for growth, although it is only one-third as effective as cystine. Lavine [1936] showed that cystine disulphoxide probably undergoes dismutative decomposition to cystine and cysteine sulphinic acid according to the reaction, $3R(SO)_2R \rightarrow RSSR + 4R(SO_2)H$, and since no enzyme present is capable of reducing the sulphoxide to the disulphide form, once the oxidation process has set in, it cannot be freely reversed. Since cysteine sulphinic acid does not support growth in place of cystine [Bennett, 1937], the tissues appear to be unable to reverse appreciably the series of reactions leading to its formation. Cystamine disulphoxide has not been tested, but our meagre knowledge of biological carboxylations does not lead to the expectation that this step is reversible. Hence we may conclude that while two pathways to taurine formation are open to cystine or to cysteine, once the initial steps are taken, reversibility is largely lost and has completely disappeared with formation of cysteic acid or taurine, and probably with cystamine disulphoxide. Experiments with tissue slices or with partially purified enzymes can scarcely present any criterion for deciding what governs a choice of pathways in the intact organism. The fact that the decarboxylation of cysteic acid must occur under anaerobic conditions unless further disruption of the molecule is to be brought about, presents an additional complicating factor. Since end-products begin to appear in the urine within a few minutes after the ingestion of cystine [Medes, 1937] the speeds of reactions in the liver or kidney must far exceed those in experiments done *in vitro*, and it is scarcely to be expected that relative rates will be maintained under conditions so unphysiological.

In all the experiments recorded above, the S recovered was only a portion of that ingested. In one case (Exp. 1, Table 1) 58 % of the ingested S was found, largely in the organic portion. In the other experiments recovery ranged from 11 to 40 %; Schmidt & Clark [1922] recovered about 58 %. White *et al.* [1937] recorded the appearance of all the S in the organic fraction of rabbit urine following ingestion of 1·58 m.equiv. of cysteic acid. This high recovery cannot be ascribed entirely to the relatively low dose, as in the experiment described in this paper, in which very small portions were ingested, total recovery amounted to 32 %. Nor can it be ascribed entirely to the fact that it was

injected, as in the experiment recorded here, in which the cysteic acid was similarly administered, only 11 % was recovered. It was hoped that in these detailed experiments some clue to the fate of the remaining S would be found, but there was no evidence of its delayed excretion either in urine or bile. Can cysteic acid be elaborated into some other S compound which is retained and utilized? Possibly the rabbit, in which 100 % was recovered, has some different mechanism or more powerful enzymes available for its conversion into taurine. However, apparently cysteic acid readily forms taurine when cholic acid is present in sufficient amounts to form taurocholic acid; hence under strictly physiological conditions, this S retention may never occur.

Summary

Cysteic acid is decarboxylated anaerobically by an enzyme located in the kidney, with production of taurine.

Under aerobic conditions, further fragmentation of the molecule occurs, with loss of the amino group.

A similar aerobic breakdown takes place in the presence of intestinal mucosa.

Because of this activity of the intestinal wall, the form in which cysteic acid is excreted after ingestion is different from that which appears after intravenous injection. The non-S fraction of the molecule has not been identified.

No evidence could be found that sulphopyruvic acid, formed in transamination reactions, contributes to sulphate formation in the tissues.

The authors are indebted to Mary Stella Cammaroti for much of the technical work, especially determinations of amino-N and carboxyl groups.

REFERENCES

Andrews, J. C. & Randall, A. [1935]. *J. clin. Invest.* **14**, 517.
Beard, H. H. [1926]. *Amer. J. Physiol.* **75**, 658.
Bennett, M. A. [1937]. *Biochem. J.* **31**, 962.
Bernheim, F. & Bernheim, M. L. C. [1939]. *J. biol. Chem.* **127**, 695.
Blaschko, H. [1942]. *J. Physiol.* **101**, 6*P*.
Cohen, P. P. [1940]. *J. biol. Chem.* **136**, 565.
Foster, M. G., Hooper, C. W. & Whipple, H. G. [1919]. *J. biol. Chem.* **38**, 421.
Gregory, R. & Pascoe, T. A. [1929]. *J. biol. Chem.* **83**, 35.
Lavine, T. F. [1936]. *J. biol. Chem.* **113**, 583.
Lewis, G. T. & Lewis, H. B. [1925]. *Proc. Soc. exp. Biol., N.Y.*, **23**, 359.
Medes, G. [1937]. *Biochem. J.* **31**, 1330.
—— [1939]. *Biochem. J.* **33**, 1559.
Medes, G. & Floyd, N. [1942]. *Biochem. J.* **36**, 259.
Schmidt, C. L. A., Adelung, E. & Watson, T. [1918]. *J. biol. Chem.* **33**, 501.
Schmidt, C. L. A. & Clark, G. W. [1922]. *J. biol. Chem.* **53**, 193.
Sealock, R. R. [1941]. *Science*, **94**, 73.
Virtue, R. W. & Doster-Virtue, M. E. [1939]. *J. biol. Chem.* **127**, 431.
White, F. R., Lewis, H. B. & White, J. [1937]. *J. biol. Chem.* **117**, 663.

100. A Note on the Estimation of the Sulphonamide Drugs by the Method of Werner

By **Colin Ashley Mawson**, *From the Pathological Laboratory, Royal Berkshire Hospital, Reading*

(*Received 18 August 1942*)

The usual methods for estimation of sulphonamide drugs in biological fluids depend upon diazotization and coupling with an amine (cf. Bratton & Marshall [1939] and Hynes [1940]). Although they are very accurate these methods call for reagents not commonly available in clinical laboratories, the solutions of which deteriorate rather rapidly. A method described by Werner [1939], using the yellow colour produced by adding *p*-dimethylaminobenzaldehyde to a solution containing the drug, avoids the inconveniences inherent in the diazotization procedure. Morris [1941] has modified Werner's method by the use of toluenesulphonic acid for protein precipitation and has introduced a citrate buffer which considerably increases the relative density of the colour and at the same time eliminates random errors which occur with the original method. However, the aldehyde reagent in Morris's method is dissolved in alcohol and it has been found that when a microcolorimeter is used fine bubbles form under the plunger which give rise to a fictitiously high result, especially if a neutral grey screen is used as a standard.

By the use of salicylsulphonic acid for protein precipitation a blood filtrate having *p*H 1·26 can be obtained which, if the aldehyde reagent is made up in aqueous solution containing sulphuric acid, gives a colour of similar relative density to that obtained in Morris's method. In the absence of alcohol no bubbles are formed.

Experimental

Reagents. (1) 30 g. 'Analar' salicylsulphonic acid made up to 100 ml. with water.

(2) 7 % aldehyde reagent. Wash 7 g. 'Analar' *p*-dimethylaminobenzaldehyde into a 100 ml. flask with 7 % *v/v* H_2SO_4 and make up to 100 ml. with 7 % H_2SO_4. Shake until dissolved, add about 0·3 g. 'Decolorizing Charcoal B.D.H.' and shake again for 2 min. Filter.

Method

Determination of sulphonamides in blood. Add 0·5 ml. blood to 6·5 ml. water, mix and leave 3 min. Add 0·5 ml. 30 % salicylsulphonic acid, shake vigorously and leave for 10 min. Filter through two filter papers folded together (Whatman No. 2, 3 or 40). If the filtrate is cloudy, return the first few ml. through the filter papers.

To 2 ml. clear filtrate add 0·5 ml. 7 % aldehyde reagent. Compare the colour produced with a standard, using a violet or blue Ilford 'Spectrum' filter on the eyepiece of the colorimeter.

Standards. The best standard to use is one of the neutral grey screens supplied by Messrs Ilford. The 0·25 screen is suitable for blanks and usually the 0·50 or 0·75 is appropriate for test solutions. If a permanent standard of this type is used a calibration curve may be constructed for each drug by addition of varying amounts to blood known to be free from sulphonamides. This may conveniently be done by replacing the 6·5 ml. water in the method by standard aqueous solutions of the drug. Owing to the low solubility of the substituted sulphonamides 10 or 20 mg. of the substance should be dissolved in the minimum of warm alcohol and transferred to 800 ml. water in a litre flask,

which is then filled to the mark with water. Using a violet 'Spectrum' filter on the eyepiece of a Klett microcolorimeter it has been found that if

$$D_v = \frac{\text{density of neutral grey screen}}{\text{cm. depth of solution}}$$

then mg. sulphapyridine/100 ml. blood $= 14{\cdot}4 \times D_v - 2{\cdot}7$. The appropriate conversion factors for other sulphonamides may be derived by direct calibration or by making the assumption (cf. Morris [1941]) that an equal amount of colour is produced by one molecule of each of the drugs.

The yellow colour of the aldehyde reagent gives rise to a considerable blank which introduces difficulties if direct comparison between coloured solutions is preferred to the use of a neutral grey standard. Satisfactory results can, however, be obtained with a sulphanilic acid solution as the standard if the calculation is carried out by means of the equation of Delory & Jacklin [1942]. If C_S and C_T are the concentrations of the substance under investigation in the standard and test solutions and R_S, R_T and R_B are the colorimeter readings of standard, test and blank respectively, the usual colorimetric calculation is given by

$$C_T = \frac{R_S}{R_T} \times C_S. \qquad \text{.....(1)}$$

The improved equation of Delory & Jacklin states that

$$C_T = \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S. \qquad \text{.....(2)}$$

The relative density of the colour produced by adding 0·5 ml. aldehyde reagent to 2 ml. of a solution of sulphanilic acid in 2 % salicylsulphonic acid has been compared with that produced by blood filtrates containing known amounts of sulphapyridine and it has been found that, for equal colour densities, mg. sulphapyridine/100 ml. blood $= 15{\cdot}4 \times$ mg. sulphanilic acid/100 ml. standard $- 0{\cdot}85$. Substituting this in equation (2)

$$C_T = 15{\cdot}4 \times \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S - 0{\cdot}85, \qquad \text{.....(3)}$$

where R_B is the reading of a blank obtained by addition of aldehyde reagent to 2 % salicylsulphonic acid and C_S is the concentration of the sulphanilic acid standard.

Table 1 shows that the results obtained by direct comparison with sulphanilic acid standards using equation (1) are unsatisfactory, especially when concentrations of sulphapyridine are small.

Table 1. *Results obtained by direct comparison using formulae based on equations (1) and (2)*

	mg. sulphapyridine/100 ml. blood	
	By direct comparison	
From reference curve	mg./100 ml. $= 15{\cdot}4 \times \frac{R_S}{R_T} \times C_S - 0{\cdot}85$ (from equation 1)	mg./100 ml. $= 15{\cdot}4 \times \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S - 0{\cdot}85$ (from equation 2)
13·7	13·4	14·0
10·4	10·8	10·2
7·2	7·6	7·2
5·0	5·5	4·9
3·1	2·8	3·2
1·6	1·9	1·4
1·2	0·3	1·1
0·8	0·2	0·9
0·1	−0·04	0·1

R_S = reading of standard. R_T = reading of test. R_B = reading of sulphanilic acid blank (4 ml. 2% salicylsulphonic acid + 1·0 ml. aldehyde reagent).

C_S = mg./100 ml. sulphanilic acid in standard. SAA = sulphanilic acid.

The use of the equation of Delory & Jacklin, however, gives good correspondence throughout the range even when the depth of colour of the standard differs very considerably from that of the test solution.

Stock standard solution. 20 mg. 'Analar' sulphanilic acid dissolved in 100 ml. 2% salicylsulphonic acid.

'Blood standard' solutions. 1 ml. stock standard diluted to 20 ml. with 2% salicylsulphonic acid.

If equation (3) is used for the calculation of results this standard is suitable for most purposes, but higher dilutions with 2% salicylsulphonic acid may be used if desired. The sulphanilic acid solutions are not subject to rapid deterioration—the 'blood standard' may be heated in a boiling water bath for 6 hr. without alteration of the density of the final yellow colour.

Estimation of total sulphonamides

If it is desired to estimate total sulphonamide the acetyl derivative may be hydrolysed with acid and the colour developed as for free sulphonamide. Reference curves must be constructed with addition to the reagents of the amount of acid used for hydrolysis.

Method. To 2 ml. blood filtrate in a graduated tube add 0·5 ml. 8% *v/v* (about 3 *N*) H_2SO_4. Shake and stopper with a cork in the side of which a small *V*-shaped notch has been cut. Heat in a gently boiling water-bath for 40 min., cool, add water to 2·5 ml. and then 0·5 ml. 7% aldehyde reagent.

In the particular case of the estimation of acetylsulphapyridine a complication arises owing to hydrolysis of the sulphapyridine itself during the heating. Furthermore, the rate of hydrolysis of free sulphapyridine differs significantly from that of sulphapyridine as liberated from the acetyl compound. Since the original free sulphapyridine is partially destroyed during hydrolysis of the acetyl compound the amount of colour it forms is less than that obtained by adding aldehyde reagent to unheated blood filtrate plus acid. The proportion destroyed under the conditions given in the method has been found to be 13%. Hence in order to calculate what proportion of the colour obtained after hydrolysis is due to the original free sulphapyridine it is necessary to multiply the density of colour obtained with unheated blood filtrate plus acid by 0·87. The remaining colour is due to hydrolysed acetylsulphapyridine, the amount of which may be derived from a reference curve constructed by means of addition of aqueous solutions of monoacetylsulphapyridine to normal blood.

The monoacetyl derivatives of the sulphonamides are easily prepared by warming with a slight excess of acetic anhydride in presence of sodium carbonate, followed by recrystallization from hot alcohol.

It should be noted that the various constants quoted above apply only to the instruments and conditions in the author's laboratory.

SUMMARY

A modification of Werner's method for the estimation of the sulphonamide drugs has been described.

The author is indebted to Dr E. C. B. Mattick of the National Institute for Research in Dairying for estimating the *p*H of a number of solutions, and also to Messrs May and Baker Ltd. for the gift of several sulphonamide drugs and a specimen of monoacetylsulphapyridine.

REFERENCES

Bratton, A. C. & Marshall, E. K. [1939]. *J. biol. Chem.* **128**, 537.
Delory, G. E. & Jacklin, J. [1942]. *Biochem. J.* **36**, 281.
Hynes, M. [1940]. *Lancet*, no. 238, 261.
Morris, C. J. O. [1941]. *Biochem. J.* **35**, 952.
Werner, A. E. A. [1939]. *Lancet*, no. 236, 18.